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REVIEW

RNA Interference. An Approach to Produce Knockout Organisms and Cell Lines

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Abstract—In various eucaryotic organisms double-stranded RNA causes effective degradation of homologous mRNA molecules by a process called RNA interference. RNA interference is a phenomenon associated with gene suppression via regulatory RNA molecules, which are common in plants, animals, and fungi. The discovery of RNA interference stimulated the development of new approaches for suppression of target gene expression, production of stable knockout cell lines and organisms, and also stimulated studies on possible intracellular functions of this phenomenon.

Key words: RNA interference, siRNA, stRNA, RISC, knockouts, Dicer, post-transcriptional gene silencing, double-stranded RNA

RNA interference rests on specific degradation of a target RNA molecule in response to the presence of double-stranded RNA with nucleotide sequence identical to that of the target RNA.

Double-stranded RNA can be endogenous or introduced into the cell from the outside. Thus, introduction into the cell of double-stranded RNA can result in a highly effective and specific suppression of the target gene expression. Because RNA interference is found in the majority of eucaryotic organisms, this approach is promising for production of functional knockout organisms and for studies on functional genomics of eucaryotes.

Before the finding of RNA interference, the technique of anti-sense RNA was mainly used to suppress gene expression. Unlike RNA interference, the technique of anti-sense RNA includes introduction into the cell of anti-sense single-stranded oligonucleotides which bind to the target RNA through production of complementary base pairs. This either activates nucleases that cut the target RNA or suppress the translation of the target mRNA preventing the movement of ribosomes over it.

At present, RNA interference is a hot topic in molecular biology. Such interest is due to both its use as an approach for production of knockouts and an attempt to determine the natural intracellular function of double-stranded RNA. The fate of double-stranded RNA introduced from the outside or synthesized inside the cell is also important.

HISTORY OF THE DISCOVERY OF RNA INTERFERENCE

Unexpected results were obtained in 1995 in experiments on *Caenorhabditis elegans* with anti-sense RNA [1]. The researchers injected into embryos of *C. elegans* anti-sense RNA to the *par-1* gene. The sense RNA corresponding to mRNA of the *par-1* gene was used as the control. The logic of the experiment was simple: the anti-sense RNA binds to mRNA of the target gene due to complementary base-pairing, and resulting double-stranded RNA molecules inhibit the protein synthesis from mRNA. The anti-sense RNA to the *par-1* gene really suppressed its expression. However, the *par-1* expression was also suppressed by introduction of the sense RNA with the nucleotide sequence identical to that of mRNA of *par-1*.

Studies on this phenomenon have shown [2] that the expression of the target mRNA molecule was directly suppressed by very low concentration of double-stranded RNA in the preparations of anti-sense and sense RNA. Just double-stranded RNA suppressed the expression of the target RNA molecule. This double-stranded RNA suppressed expression of the target gene significantly more effectively than the corresponding sense and anti-sense RNA.

Even the first studies on RNA interference showed its main features: specificity [2, 3] (expression is sup-

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pressed only of the gene with the nucleotide sequence completely corresponding to the nucleotide sequence of the double-stranded RNA injected); efficiency (90% and more [2, 4, 5]); RNA interference occurs post-transcriptionally and is associated with directed degradation of the target mRNA molecule [2, 3, 6-8]; the effect of RNA interference in any region of the *C. elegans* body can be extended onto the whole organism of the nematode, and, moreover, be inherited by offspring [2, 9]. These features suggest the existence of a certain mechanism of RNA interference. Because RNA interference can be extended into the whole organism and be inherited by the offspring and also because the RNA interference effect can be obtained by injection of only a few molecules of double-stranded RNA per cell [2, 6], it was suggested that a catalytic mechanism should amplify the effect of RNA interference. What is the mechanism of RNA interference?

MECHANISM OF RNA INTERFERENCE

Experiments on cell-free systems were significant for development of current ideas on the mechanism of RNA interference [10-12]. The use of these systems revealed the main stages of RNA interference and the fate of double-stranded RNA injected into the cell.

In the first system, RNA interference was studied *in vitro* in extracts of *Drosophila melanogaster* embryos at the stage of the syncytial blastoderm [10]. On addition of radiolabeled double-stranded RNA (~500 b.p.) and the subsequent incubation, the radioactivity was located in fragments of double-stranded RNA of 21-23 b.p. size [11]. These findings suggested that the incubation of double-stranded RNA with the extract from *D. melanogaster* embryos resulted in its nonspecific processing with production of double-stranded RNA molecules of 21-23 b.p. size. These molecules were called short interfering RNA (siRNA). This process needs no target RNA, and its efficiency depends on ATP (in the presence of ATP the rate of siRNA production is increased sixfold) [11].

The resulting siRNA contained 5′-phosphate and 3′-hydroxyl groups on the ends of the molecule and had overhanging single-stranded 3′-ends 2 b.p. in size [13]. Similar products of cleavage and the substrate specificity characterize nucleases of the RNase III family. It was suggested that some nucleases of this family should catalyze fragmentation of long molecules of double-stranded RNA with production of siRNA [13].

Several nucleases from *D. melanogaster* with domains specific for the RNase III family were tested. Only one protein called Dicer displayed the corresponding activity to double-stranded RNA [14]. Dicer contains two regions homologous to those of other RNases III and the C-terminal RNA-binding site and the N-terminal helicase region. Treatment of double-stranded RNA with immunoprecipitated Dicer resulted in fragmentation of long molecules of double-stranded RNA onto siRNA 22 b.p. in size. This fragmentation was ATP-dependent. The importance of Dicer for RNA interference was also shown by *in vivo* experiments. Injection of double-stranded RNA corresponding to the sequence of Dicer into the S2 cell line disturbed RNA interference: under these conditions the GFP expression was suppressed insignificantly by corresponding double-stranded RNA [14]. The incomplete suppression of RNA interference suggested that this process should be realized by more than one mechanism. Thus, on entrance into the cell long molecules of double-stranded RNA are cleaved by Dicer with production of siRNA of 21-23 b.p. size, and hydrolysis of ATP is required. Just the nuclease activity of both recombinant and endogenous human Dicer requires no ATP [15, 16]. The helicase domain of Dicer is suggested to have the ATPase activity, and it should be involved in untwisting of the RNA duplex or in promotion of Dicer over double-stranded RNA and regulation of siRNA production. On the other hand, ATP can regulate the binding of Dicer to double-stranded RNA or change the activity of RNase III-like domains of Dicer [17].

Further studies have shown siRNAs to be components of the nuclease complex that mediates the specific degradation of the target RNA [12]. The high specificity of such complex is determined by siRNA due to complementary pairing of bases of the siRNA anti-sense chain and target RNA. Thus, the nuclease activity can select its target among the great abundance of intracellular RNAs.

In extracts of S2 cells from *D. melanogaster* pretransformed with double-stranded RNA, an endonuclease complex was found and partially purified. This complex was specific to mRNA with sequence identical to that of the earlier injected double-stranded RNA. This complex called RISC (RNA-induced silencing complex) [12] includes protein and RNA: on treatment of RISC with micrococcal nuclease (which cleaves RNA and DNA) the extracts lost the ability to degrade the target RNA. DNase I had no effect on the RISC functions [12]. Further analysis resulted in identification of some proteins of RISC. Thus, the RISC isolated from the S2 cell line from *D. melanogaster* includes a protein Argonaute-2 (Ago-2) [18]. This protein is from a large protein family called *Argonaute* or PPD. Proteins of this family are specified by the presence of PAZ and C-terminal Piwi domains with unknown functions [19, 20]. Dicer also contains the PAZ domain [19]. In particular, the PAZ domain is likely to be necessary for protein-protein interactions, therefore, Dicer and Argonaute-2 interact in the S2 cells [18]. The interaction of these proteins is likely to promote the entrance of siRNA into the RISC. Studies of two other PPD-proteins, Qde-2 and Rde-1, have shown that they are constituents of the RISC from *Neurospora crassa* [21] and *C. elegans* [22], respectively.

From extracts of HeLa S100 cells a human RISC was purified with molecular weight between 90 and 160 kD

[23]. By mass-spectrometry two proteins, eIF2C1 and eIF2C2, were identified in the human RISC that belong to the *Argonaute* family [23]. Antibodies to eIF2C2 caused immunoprecipitation of the RISC activity from extracts of HeLa S100 cells [24]. The RISC activity was also immunoprecipitated with monoclonal antibodies to Gemin3 and Gemin4 [24]. Note that Gemin3 is suggested to be an RNA-helicase including the DEAD region. Thus, at least four proteins, eIF2C1, eIF2C2, Gemin3, and Gemin4, are constituents of the human RISC.

Based on these data, the following model of RNA interference was designed (Fig. 1). On entrance into the cell, double-stranded RNA is cleaved by Dicer with production of siRNA. These siRNA became a component of

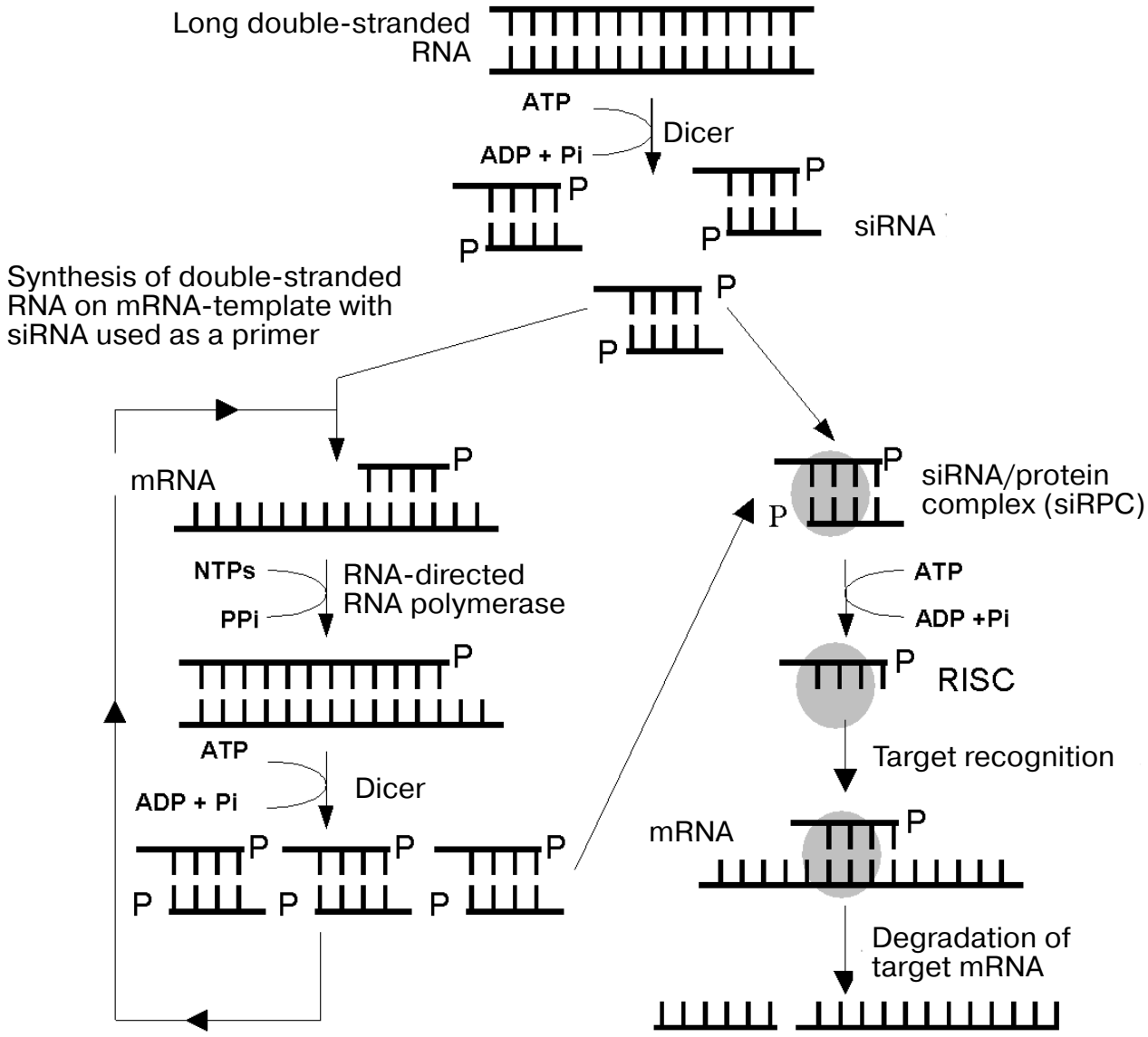

Fig. 1. Scheme of the mechanism of RNA interference in *C. elegans*. In the presence of Dicer, double-stranded RNA is cleaved with production of siRNA. The siRNA is a constituent of RISC (RNA-induced silencing complex), and siRNA of 21-23 b.p. size direct the specific activity of RISC to target mRNA and thus determine its specific degradation. The RNA interference effect is amplified as a result of processing of long molecules of double-stranded RNA with production of a set of siRNAs capable of inducing a specific degradation of their targets. On the other hand, the primary siRNA can act as a primer for synthesis of double-stranded RNA on the target mRNA template under the influence of RNA-directed RNA polymerase. The resulting double-stranded RNA is cut by Dicer with production of the secondary siRNA, which acts again as a primer for synthesis of double-stranded RNA on mRNA template. Thus, Dicer is required not only for production of siRNA, but it can cause the degradation of the target mRNA converted to the double-stranded form under the influence of RNA-directed RNA polymerase (after [60]). In this and other figures proteins in RISC are shown by darkened circles.

the RISC, and siRNA of 21-23 b.p. size direct the endonuclease activity of the RISC to the target RNA and, thus, provide for the specific degradation only of the target RNA molecule. Note, that the RISC causes the cleavage not only of the sense mRNA but also of its anti-sense RNA. Such an activity of the RISC can be explained either by the presence in it of double-stranded RNA [25], or by existence in the extract of two complexes: one with the sense RNA (this complex is responsible for degradation of the anti-sense RNA), and the other complex with the anti-sense RNA (responsible for degradation of the sense RNA) [13].

In extracts from HeLa S100 cells and *D. melanogaster* embryos, RISC was shown to contain single-stranded RNA [23, 26]. Incubation of both anti-sense single-stranded RNA of 15-29 b.p. size and siRNA in extracts from HeLa S100 cells and *D. melanogaster* embryos resulted in appearance of the RISC activity specific for the target RNA [23, 26]. Single-stranded RNA was also shown to induce the RNA interference response *in vivo* by transfection of such RNA to HeLa S100 cells [23]. However, siRNA was more effective and acted in lower concentrations than single-stranded RNA. Consequently, there is a special mechanism specific for siRNA and responsible for incorporation of one of siRNA chain into RISC. It is known that siRNA, before induction of the RNA interference effect, is separated onto two chains, and this depends on energy from ATP [27]. Possibly, siRNA was separated under the influence of ATP-dependent RNA helicases [28-30].

Enzymes involved in RNA interference are located in the cytoplasm. Only cytoplasmic extracts from HeLa S100 cells displayed the RISC activity, no such activity being found in nuclear extracts from these cells [23, 24]. Dicer is also a cytoplasmic protein [31]. The recombinant human Dicer expressed in mammalian cells is located in endoplasmic reticulum [15]. Dicer and RISC seem to interact in the cytoplasm. Dicer was shown to interact with Argonaute-2, which is a constituent of RISC from *D. melanogaster* [18].

RNA interference not only causes degradation of the target RNA located in the cytoplasm; during the export from the nucleus, the target mRNA is also destroyed due to RNA interference [32], whereas the nuclear RNA is resistant to the degradation mediated by RNA interference. Fluorescently-labeled siRNA is located immediately close to the nucleus [33]. The authors suggested that siRNA should be concentrated around nuclear pores, and they should "scan" mRNA that is transported into the cytoplasm. If the sequence of target mRNA is detected, it is cleaved [33].

However, this mechanism fails to explain all features of RNA interference. Thus, it is unclear why RNA interference that has arisen in a region of the *C. elegans* body is propagated onto the whole animal and is inherited [2, 9]. How can it be explained that a small number of double-stranded RNA molecules per cell (~25 molecules) can suppress the expression of more than 1000 molecules of the target mRNA [2, 6]? Obviously, *C. elegans* has to possess systems for amplification of the RNA interference effect and its intercellular transport. This amplification can partially be a consequence of processing of long molecules of double-stranded RNA that results in production of a set of siRNA capable to specifically degrading their targets. Obviously, such an amplification cannot be the only mechanism explaining all features of RNA interference. Genetic studies have shown that mutations in genes whose products are homologous to RNA-directed RNA polymerases suppress RNA interference [34-36]. These findings suggest a significant role of these enzymes in the realization of RNA interference.

Based on data for extracts from *D. melanogaster* embryos, a model was proposed with siRNA as a primer for the synthesis of double-stranded RNA on the template of the target mRNA [25]. It seems that under the influence of ATP-dependent RNA helicases, siRNA is separated onto two chains, one of which plays the role of a primer. Thus, organisms with mutant genes of RNA helicases are unable to realize RNA interference [28-30].

Treatment of extracts with preincubated double-stranded RNA with micrococcal nuclease resulted in a fraction enriched with siRNA (proteins in RISC protect siRNA from cleavage by the nuclease). The purified siRNA failed to specifically degrade the target mRNA. However, its RNA interference activity was recovered by treatment with alkaline phosphatase. Products of RNA cleavage under the influence of micrococcal nuclease were mono- or oligoribonucleotides with 3′-phosphate groups on the ends. Thus, the presence of phosphate on the 3′-end of siRNA suppressed RNA interference, whereas to realize RNA interference the presence of 3′-terminal hydroxyl group was necessary to promote siRNA to play the role of a primer for RNA-directed RNA polymerase.

Incubation of radiolabeled siRNA in extracts with the target mRNA resulted in production of double-stranded RNA corresponding to the target mRNA. Increase in the incubation time was accompanied by a decrease in the amount of full-size RNA and accumulation of the newly produced secondary siRNAs. Based on these data, a model of amplification of the RNA interference effect was designed which was called degradative-PCR [25]. Exogenous or endogenous double-stranded RNA is cut in the cell by Dicer with production of the primary siRNA which in its turn can act as a primer for synthesis of double-stranded RNA on the target mRNA template with the involvement of RNA-directed RNA polymerase. The resulting double-stranded RNA is cut by Dicer with production of the secondary siRNA, which also acts as a primer for synthesis of double-stranded RNA on the mRNA template (Fig. 1). Thus, Dicer is required not only for production of siRNAs but it also

causes the purposeful degradation of mRNA preliminarily converted to the double-stranded form under the influence of RNA-directed RNA polymerase [25]. For accumulation of a sufficient amount of siRNA in *Dictyostelium discoideum*, both the target mRNA and the gene encoding the RNA-directed RNA polymerase were required [37]. This finding confirms the validity of the above-presented model.

The possible roles of the RNA-directed RNA polymerase activity and siRNA in the amplification of the RNA interference effect were also shown by a refined experiment on *C. elegans* [38]. The nematodes were injected with constructs expressing two different target RNA molecules: one included the sequence X (X-RNA) and the other included the sequence X fused with the 5′-end of the sequence Y (XY-RNA). RNA interference was realized by injection of double-stranded RNA that corresponded to the Y-sequence. If siRNA really acts as a primer for synthesis of RNA on the mRNA template, then its cleavage after the synthesis of double-stranded XY-RNA under the influence of DCR-1 (a homolog of Dicer from *D. melanogaster*) would result in production not only of siRNA corresponding to the sequence Y, but also of siRNA corresponding to the sequence X. The presence of siRNA homologous to the sequence X would result in degradation of X-RNA. The specific degradation of X-RNA was actually found experimentally.

The above-described model of the RNA interference mechanism based on the synthesis of a new RNA chain on the target RNA template with involvement of RNA-directed RNA polymerase and siRNA as a primer is obviously true for such organisms as *C. elegans*, *Dictyostelium discoideum*, *N. crassa*, and *Arabidopsis thaliana* [39]. Homologs of RNA-directed RNA polymerase are found in each of these organisms, and genes encoding these enzymes are quite necessary for RNA interference. No homologs of RNA-directed RNA polymerases are found in *D. melanogaster* and mammals. Moreover, inhibition of the 3′-OH-group of siRNA failed to disturb RNA interference in extracts from HeLa S100 and *D. melanogaster* embryos [23, 26, 40]. Therefore, the presence of a 3′-OH-group in siRNA is not imperative, and, consequently, siRNA cannot play the role of primers for synthesis of double-stranded RNA in human and *D. melanogaster* cells.

The possibility to selectively suppress expression only of one of several protein isoforms expressed concurrently in mammalian systems using siRNA specific for this isoform [41] suggests a failure of the degradative-PCR model for mammalian cells. Endonuclease cleaves the target RNA in extracts from HeLa S100 and *D. melanogaster* embryos with the mature RISC in the absence of the energy of ATP [24]. Thus, RNA interference in the systems studied occurs without mechanisms associated with such energy-consuming processes as synthesis of a new RNA on the target RNA template and a subsequent degradation of the resulting double-stranded RNA under the influence of Dicer.

Although the RNA interference effect is not amplified in human and insect cells, it is extremely effective. Each RISC produced causes a specific cleavage of ~10 molecules of the target RNA [24]. Thus, RISC is a true enzyme responsible for catalysis of several rounds of cleavage of the target RNA.

Biochemical studies on RNA interference *in vitro* resulted in the concept on the main stages of this process, whereas the genetic analysis of mutants provided important information about its contributors. By studies on *C. elegans*, *A. thaliana*, *D. melanogaster*, and *N. crassa* mutants deficient in RNA interference and related mechanisms (post-transcriptional gene silencing (PTGS) in plants and quelling in fungi), a significant number of genes and their products necessary for RNA interference were found. RNA interference occurs with the involvement of proteins of the PPD family [42, 43]: RDE-1 (*C. elegans*) [44], Qde-2 (*N. crassa*) [45, 46], Ago-1 (*A. thaliana*) [42, 43]. Note, that proteins of the PPD family are homologous to the eIF2c from rabbit reticulocytes. The Mut-7 protein from *C. elegans* required for RNA interference is a homolog of RNase D from *E. coli* [47]. Moreover, the involvement has also been shown of ATP-dependent helicases: Qde-3 (*N. crassa*) is homologous to DNA helicase RecQ from *E. coli* [48]; SMG-2 (*C. elegans*) [28] and SDE3 (*A. thaliana*) [29] are homologous to Upf1p- and SMG-2-like helicases; Mut-6 (*Chlamydomonas reinhardtii*) [30] is homologous to RNA helicases containing the DEAH-domain. Analysis of the mutants has also shown the role of RNA-directed RNA polymerases in RNA interference. Thus, organisms unable to realize RNA interference having mutants in genes homologous to RNA-directed RNA polymerases EGO-1 and RRF-1 (*C. elegans*) [49], Qde-1 (*N. crassa*) [34], and SDE1/SGS2 (*A. thaliana*) [35, 36] have been found.

The immediate role of most of these proteins in RNA interference is unknown. It seems that the mechanism of RNA interference will be successfully understood as a result of genetic and biochemical approaches combined.

RNA INTERFERENCE AS AN APPROACH IN MOLECULAR BIOLOGY

After double-stranded RNA was found to specifically and effectively suppress gene expression, RNA interference became a standard experimental procedure for preparation of functional knockout organisms and a significant method of functional genomics of eucaryotes [50]. Knockouts of *C. elegans*, *D. melanogaster*, and *Trypanosoma* produced by RNA interference displayed the same phenotypes as knockouts produced by conven-

tional methods. An additional advantage of RNA interference, besides its efficiency and specificity, is simplicity and time-saving in production of knockouts.

There are several strategies for producing RNA interference (Fig. 2). The first group of approaches is based on injection of double-stranded RNA into organisms or cell lines. Double-stranded RNA was earlier prepared by cloning of the corresponding DNA insertion into vector which contained sequences of phage promotors (T7, T3, or SP6) from both sides of the site cloned [2, 51-53]. The resulting plasmid was cleaved with a restrictase specific for the site remote from the insertion and used to prepare RNA products via *in vitro* transcription. The transcript contained sense and anti-sense RNA that constituted double-stranded RNA (Fig. 2a). This method is used to produce relatively long molecules of double-stranded RNA. Short molecules of double-stranded RNA are produced similarly by PCR [10-12, 14, 31, 54]. With primers containing phage promotor sequences on the 5′-ends, certain regions of DNA can be amplified; to produce double-stranded RNA the amplified DNA is *in vitro* transcribed (Fig. 2b). The resulting double-stranded RNA is introduced into *C. elegans* either by injection of a solution containing this double-stranded RNA [2] or by a simple placing of the animal into this solution [55].

RNA interference is often realized in *C. elegans* as follows [51, 53]. The DNA insertion corresponding to double-stranded RNA is cloned into a vector that contains two T7 promotors from both sides of the site cloned. With this vector *E. coli* is transformed, resulting in bacterial cells expressing double-stranded RNA; *C. elegans* is grown in medium containing these *E. coli* cells as the source of nutrition; the bacteria are digested in the intestine, and released molecules of double-stranded RNA pass through the intestinal wall into the body cavity and thus extend the specific RNA interference effect over the whole organism (Fig. 2c).

Other methods to realize RNA interference are based on production of stable knockout organisms and cell lines by introduction of constructions expressing long double-stranded RNA. A number of such constructions were used to produce stable knockouts of *C. elegans*, *D. melanogaster*, and *Trypanosoma*. The first type constructions with the insertion flanked by inverted promotors were already considered by us; unlike the above-described vector, promotors and other elements responsible for the control of expression, replication, and selection are chosen with respect to specificity of the cells of the organism under study (Fig. 2f). The second type constructions contain one promotor but the insertion is cloned in the vec-

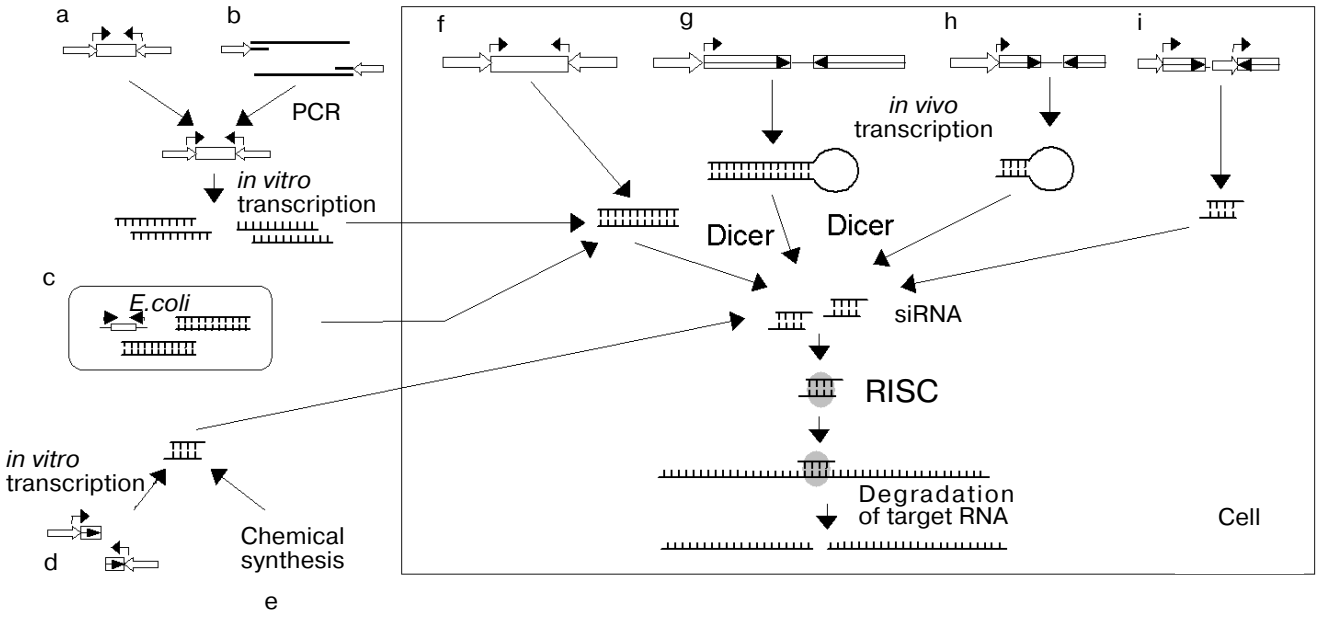

Fig. 2. Different approaches used for the target degradation of mRNA by RNA interference. All available methods are divided into two groups. In the first group long molecules of double-stranded RNA (a, b) or siRNA (d, e) are injected into the organism or cells. Another group of methods is based on production of transformants which include a vector expressing either long double-stranded RNA (f, g) or siRNA (h, i). This approach is advantageous because it provides for a stable suppression of gene expression in a definite tissue and at a definite developmental stage. A special case is presented by RNA interference in *C. elegans* that is realized by addition into the culture medium of bacteria that contain a vector expressing double-stranded RNA (c) (explanation in the text). Promotors are presented as hollow arrows that show the direction of transcription.

tor in such a way that the RNA transcribed forms a hairpin structure with a stem corresponding to necessary double-stranded RNA [56-58]. The insertion in this construction forms a palindrome interrupted in the middle with a sequence of the spacer (Fig. 2g).

By the methods described, many *C. elegans* and *D. melanogaster* knockouts of various genes were produced. However, the use of RNA interference is especially interesting for studies on functional genomics of *C. elegans* [59, 60]. These studies resulted in libraries of *E. coli* clones that produced individual double-stranded RNA each of which was homologous to a certain gene of *C. elegans*. These libraries were used to produce by RNA interference, libraries of *C. elegans* where each organism carries a certain gene suppressed. The resulting library was analyzed by parameters of disorders in the development and cell division, and the role of genes located in chromosomes I and III of *C. elegans* was established.

A similar approach was recently used for the larger-scale functional study on the *C. elegans* genome that included inactivation and analysis of 16,757 genes, which is more than 86% of the supposed number of 19,427 genes in the genome. This study resulted in a methodical analysis of bonds between the nucleotide sequences of the genes, their functions, and chromosomal location, and this provided for an approach to such a significant problem of functional genomics as the global organization of gene functions in the genome of multicellular organisms [61].

The significant success in achieving RNA interference in *C. elegans* and *D. melanogaster* stimulated searches for such systems in mammalian cells. The main difficulty was the powerful antiviral system of mammals associated with induction of interferon. On entrance into the cell, long molecules of double-stranded RNA induce a nonspecific suppression of translation and nonspecific degradation of intracellular RNA by activation of the double-stranded RNA-dependent kinase PKR (dsRNA-dependent protein kinase) and 2′,5′-oligoadenylate synthetase. The latter is responsible for synthesis of short 2′,5′-oligoadenylates which in turn activate the 2′,5′-dependent RNase L that catalyzes nonspecific degradation of intracellular RNA [62]. To achieve RNA interference in mammalian cells, it was necessary to bypass the defense systems of the body.

Specific RNA interference in mammals was first obtained by injection of long double-stranded RNA (~500 b.p.) into mouse embryos at the early developmental stages [52]. RNA interference was also achieved in the culture of embryonal stem cells and in cell lines P19 and F9 of mouse embryonal teratocarcinoma [31, 54]. The injection into the teratocarcinoma cells of constructions expressing double-stranded RNA as a hairpin with a loop (Fig. 2g) resulted in a stable suppression of gene expression [54]. It seems that the specific RNA-interference effect in the undifferentiated embryonal cells was achieved due to their lacking of some enzymes involved in the response to intracellular double-stranded RNA.

Two main strategies are used to achieve RNA interference in mammalian somatic cells that have the defense system. The first strategy is based on the double-stranded RNA minimal length of 30 b.p. capable of triggering the antiviral interferon system, whereas the length of siRNA is 21-23 b.p. Thus, the introduction into the cells of siRNA with liposomes promotes, on one hand, suppression of the target gene expression and, on the other hand, bypass of the intracellular defense systems [13, 63, 64]. At present, such an approach seems to be the most effective [64]. Because RNA interference can be obtained by injection of siRNA into the cells, a question arises of the functional anatomy of siRNA. What structure has siRNA to possess to provide for the highest and most specific RNA interference effect? Although this problem has been mainly studied on extracts from *D. melanogaster* embryos [65], the findings correlate well with data on mammalian cell lines. siRNA of 21 b.p. size with single-stranded overhanging 3′-ends of 2 b.p. length was the most effective. The sequence of the first four nucleotides from the 3′-end is not highly conserved. Moreover, ribonucleotides located on the overhanging 3′-end can be replaced by deoxyribonucleotides without decreasing the RNA interference activity. This approach is used to increase the stability of siRNA, because the presence of deoxyribonucleotides on the 3′-ends increases their resistance to RNases. To achieve RNA interference, siRNA can be chemically synthesized or *in vitro* transcribed from oligonucleotide templates containing the T7-promotor [66, 67] (Fig. 2, e and d, correspondingly). The sense and anti-sense RNA chains are designed to promote the production during annealing of double-stranded siRNAs with overhanging 3′-ends.

Alternatively, siRNA is prepared by cleavage of large molecules of double-stranded RNA with RNase III from *E. coli* that results in production of so-called endoribonuclease-prepared siRNA (esiRNA) which effectively can suppress gene expression in mammalian cell lines and mouse embryos [68, 69]. Another possible strategy is based on achieving RNA interference in somatic cell lines unable to activate the interferon antiviral system. Such cell lines can contain mutations in genes which encode enzymes responsible for nonspecific response to double-stranded RNA, or genetic constructions that express inhibitors of these enzymes of virus origin (such as adenovirus VA RNA which binds to PKR but does not activate it, or K3L of the variolovaccine virus which binds to PKR and inhibits it) [54, 70, 71].

Notwithstanding certain difficulties in production of stable knockouts in mammalian somatic cell lines, this problem has been recently solved by creation of new type vectors able to synthesize siRNA in mammalian cells [66, 72-77]. These vectors are specified by the presence of DNA insertion controlled by the promotor for RNA-polymerase III. The choice of RNA-polymerase III was

reasoned by its unique ability to synthesize short RNA molecules whose size was clearly determined by the terminal sequence of DNA of 4-5 thymidine nucleotides [78]. By this approach short molecules of siRNA of strictly determined size can be synthesized. In the vectors for siRNA expression U6 and H1 promotors of RNA polymerase III are used, because promotor sequences of snRNA (small nuclear RNA) *U6* and RNA *H1* genes of RNase P are entirely located before the point of initiation of transcription [79].

There are two approaches for construction of vectors that express siRNA based on the U6- and H1-promotors. In the first type vectors the sense and anti-sense chains constituting the siRNA duplex are transcribed from two individual promotors (Fig. 2i) [73, 77]. The second type vectors contain as insertions two inverted repeats of ~21 b.p. size separated by a spacer of 3-9 b.p. The expression of such vector results in an RNA product that forms a hairpin-like structure with a stem corresponding to the double-stranded RNA required (Fig. 2h). This precursor is converted to siRNA by Dicer [72, 74-76].

The above-described approaches are used to construct retroviral vectors expressing siRNA [80-82]. Advantages of retroviral systems are obvious. Only a few somatic cell lines can be effectively transformed with plasmid vectors, and transformation of embryonal cell lines is virtually impossible. The use of siRNA-expressing systems based on retroviral vectors allows these problems to be solved. Retroviral systems are significantly more effective in the transfer of genetic constructions into various cell lines. Moreover, the ability of retroviruses to integrate into the genome and penetrate into embryonal cell lines provide for production of knockout organisms that contain siRNA-expressing constructions integrated into chromosomes. This significantly decreases the cost of knockout organisms and reduces the time of their production.

Due to the high specificity of RNA interference and efficiency of retroviral systems, the siRNA-expressing retroviral vectors seem to be a promising tool for gene therapy. In particular, such systems allow oncogene expression to be suppressed [82]. Another field of possible application of these systems is the treatment of persistent viral infections.

INTRACELLULAR FUNCTIONS OF RNA INTERFERENCE

RNA interference has been found in various eucaryotic organisms including insects [6, 7], nematodes [2], trypanosomes [4], planaria [8], hydra [5], mice [52], human cell lines [63, 64], plants [83-85], and *N. crassa* [34, 48, 86]. Such a wide distribution of RNA interference suggests its importance and functional diversity in eucaryotes.

Post-transcriptional gene silencing (PTGS) is a mechanism relative to RNA interference and responsible for antiviral defense in plants. The PTGS phenomenon was found during experiments on production of transgenic plants. The introduction into a plant of a vector expressing a product identical to that of the intracellular gene resulted completely inhibited the expression of both endogenous and exogenous genes [83-85]. This phenomenon was called cosuppression, or PTGS [87, 88]. The actively expressing transgene is supposed to transcribe aberrant RNA (aRNA) [87]. Intracellular plant RNA-directed RNA polymerase uses aRNA as a template for synthesis of double-stranded RNA [89]. This double-stranded RNA is processed with production of siRNA of 25 b.p. size [90] under the influence of enzymes which seem to be similar to Dicer. siRNA forms a RISC-like complex and directs its endonuclease activity to the target mRNA, and this results in specific degradation of mRNA of both transgene and its intracellular analog [89].

PTGS and RNA interference are related processes: in both cases the involvement of double-stranded RNA is crucial; enzymes involved in these processes are highly homologous [28, 29, 34-36, 42-45, 49]; the main stages of these processes also seem to be similar [87].

PTGS can be initiated not only by actively expressed transgenes but also by viruses that contain in their genome sequences homologous to genes of the cell [91, 92]. Due to PTGS, mRNA of the cell gene and viral RNA are specifically degraded; therefore, the virus in such cells is eliminated. This principle is fundamental for practical application of PTGS, in particular, the production of virus-resistant plants. For this purpose transgenic plants are produced which express a product of the virus-specific gene, such as the virus capsid protein. On entrance into the cells of such plant, the virus cannot realize its functions because the virus-specific RNA and mRNA of the transgene induce the mutual cosuppression [91, 92].

PTGS can also be induced by viral infection in the absence of any homology between the virus and plant genomes [93-96]. Thus, viruses can concurrently initiate PTGS and be its target. It seems that PTGS plays an important role in the recognition and elimination of heterologous nucleic acid and is involved in plant antiviral defense.

The involvement of PTGS in the plant antiviral defense is, in particular, strongly evidenced by detection of virus genes the products of which, on one hand, determine the virus pathogenicity and, on the other hand, are specific inhibitors of PTGS [97-100] that is exemplified by Hc-proteases (HcPro) of potiviruses, in particular, of the potato virus Y (PVY) and the protein 2b of the cucumber mosaic virus (CMV).

RNA interference controls mobilization of transposons. All natural isolates of *C. elegans* contain transposons. Some *C. elegans* strains are characterized by high

mobilization activity of transposons in the embryonal line cells, whereas other strains have no highly active transposons in such cells. These strains are genetically different. Mutants mut-4, mut-5, and mut-6 provide for mobilization only of the transposon Tc1 [26]. Mutations mut-7 and mut-2 are responsible for mobilization of a number of transposons including Tc1, Tc3, Tc4, and Tc5 [47, 101-103]. These data suggest the existence of a unified control system of transposon mobilization, because mutation of only one gene (mut-7 or mut-2) results in activation of different transposons. RNA interference was proposed to be such a system. This hypothesis is supported by the following data: mutants mut-7 and mut-2, which are specified by the active mobilization of transposons, are unable to realize RNA interference [47] and, by contrast, some mutants (such as rde-2 and rde-3) unable for RNA interference are very active in transpositions [44].

Based on these data, a model of control of transposon mobilization was designed with RNA interference as a nonspecific suppressor of their activity [47].

Transposons are transferred under the influence of the enzyme transposase. The more often are their transfers, the higher is the probability that the transposon will be located immediately near an active promotor. The transposon can be oriented with respect to the promotor to promote transcription of the anti-sense RNA of transposase. The interaction of anti-sense and sense RNA of transposase can produce double-stranded RNA which will induce (due to RNA interference) specific degradation of mRNA of transposase and thus suppress the transposon mobilization. Because RNA interference in not specific relative to double-stranded RNA, this mechanism can be responsible for suppression of many transposons.

These data can be also explained otherwise [47]. Because many transposons (including Tc1, Tc3, Tc4, Tc5) contain on their ends inverted repeats, RNA produced during the transcription of these repeats can easily form double-stranded RNA that can trigger RNA interference and a subsequent suppressive effect.

Dicer is involved in RNA interference and maturation of stRNA. Two stRNA (small temporal RNA), lin-4 and let-7, are single-stranded molecules of 21 b.p. size which regulate development [104-106]. Homologs of let-7 have been found in various organisms, including *C. elegans*, *D. melanogaster*, and humans [107]. Similarly to many other small RNAs, stRNA is not translated, and its effects are realized on the level of RNA. Effects of let-7 and lin-4 are displayed on the post-translational level. Unlike siRNA which induces degradation of the target mRNA [13], the let-7 and lin-4 transcripts bind to the 3′-untranslatable region of their target lin-41, lin28, and lin-14 mRNA by pairing complementary bases and prevent their translation [104, 105, 108-115]. It is known that let-7 is produced from the precursor pre-let-7 RNA of ~75 b.p. size [17, 107]. This precursor contains internal complementary regions on the ends of its molecule, and pre-let-7 RNA forms a hairpin-like structure with a stem of double-stranded RNA, with one chain corresponding to mature let-7. This precursor penetrates through nuclear pores into the cytoplasm and there it is processed and converted to mature let-7 RNA [116].

Pre-let-7 RNA with its double-stranded stem is a potential target for RNA interference [11, 12, 14, 90]. Dicer was found to be a key enzyme responsible for conversion of pre-let-7 into mature let-7 [17, 117, 118].

The involvement of Dicer in the processing of pre-let-7 is suggested based on the following findings. The maturation of pre-let-7 results in a molecule of 21 b.p. size [107], and the same size is specific for siRNA [11, 13] which is produced by the Dicer-dependent cleavage. The processing of pre-let-7 requires ATP [17], and Dicer is an ATP-dependent enzyme [14]. The structure of the resulting let-7 ends and the substrate specificity suggest that the processing of pre-let-7 occurs under the influence of an enzyme from the RNase III family [98], which also includes Dicer. Immunoprecipitates of Dicer from *D. melanogaster* embryos induce the processing of pre-let-7 which results in mature let-7-mRNA [118]. Other convincing data have been obtained in *in vivo* experiments. Thus, in HeLa cells pretransfected with siRNA corresponding to the human Dicer (to suppress the expression of Dicer by RNA interference) pre-let-7 was markedly accumulated along with a complete absence of mature let-7 [17]. Similar results were obtained on *C. elegans* mutants by the *dcr-1* gene (an analog of Dicer from *D. melanogaster*): accumulation of the precursor and a significant decrease in the amount of mature let-7-mRNA [117, 118]. It is interesting that *dcr-1* mutants of *C. elegans* manifested the same disorders in development as organisms with mutations in the let-7/lin-41 pathway [107, 117, 118].

Thus, at least one component necessary for RNA interference, Dicer, plays an important role in production of mature stRNA molecules involved in regulation of the development (Fig. 3). In the case of RNA interference and also at the processing of pre-stRNA, double-stranded RNA is a substrate of Dicer, but the resulting products are different: the production of siRNA is associated with symmetrical cleavage of double-stranded RNA, whereas the production of stRNA is preceded by cleavage only of one chain of pre-stRNA. Obviously, mechanisms of the cleavage of double-stranded RNA with endonuclease are different during RNA interference and processing of stRNA. This difference can be partially due to the secondary structure of pre-stRNA, formation of an internal loop in the 5′-region of the cleavage with endonuclease, and protein or other factors that can recognize such structures. This role can be played by RDE-1 and ALG-1/ALG-2 proteins of *C. elegans*. The mutants deprived of the functional RDE-1 are unable to realize RNA interference [44] but are normal in other

aspects; nematodes with mutations in ALG-1/ALG-2 can realize RNA interference but have disorders in development: they are unable to produce mature lin-4 and let-7 [117]. RDE-1 and ALG-1/ALG-2 belong to the same family of PPD proteins that includes proteins containing Paz and Piwi regions [42, 43]. Some members of this family seem to be involved in determination of processing of double-stranded RNA, with production of siRNA or stRNA.

Note that let-7 and lin-4 stRNA are summits of an iceberg which includes hundreds of types of so-called micro-RNA of 21 b.p. size found in various organisms [116, 119-121]. Various micro-RNAs seem to be also synthesized as precursors capable of producing hairpin-like

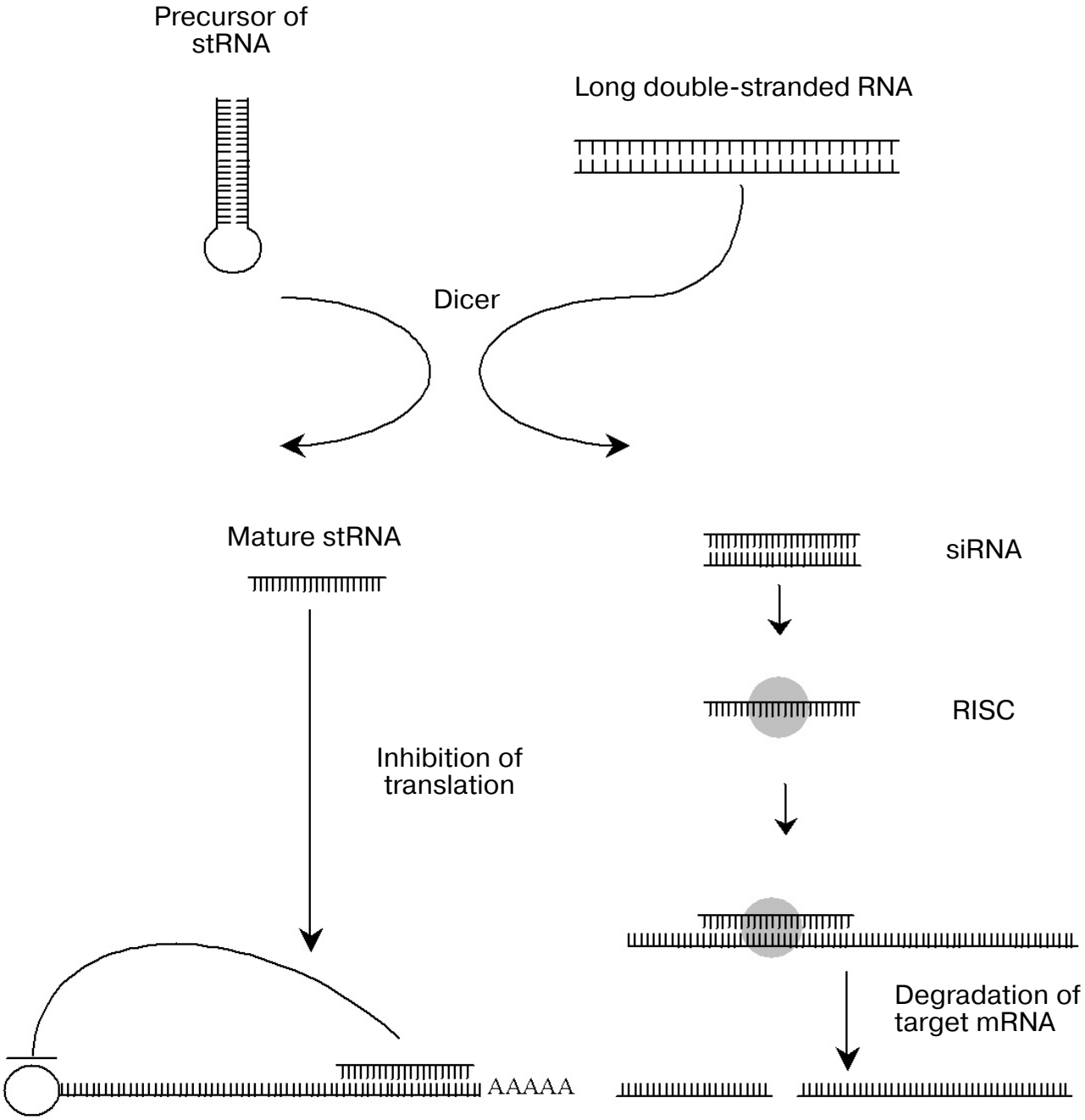

Fig. 3. Role of Dicer in the post-transcriptional regulation of gene expression. Dicer is involved in at least two systems responsible for regulation of gene expression with the involvement of regulatory RNA: it catalyzes nonspecific cleavage of long molecules of double-stranded RNA with production of siRNA and is necessary for production of mature stRNA from the precursors.

structures. Thus, Dicer is an important player not only during RNA interference but also in regulatory mechanisms that seem to involve micro-RNA.

Role of RNA interference in provision of normal spermatogenesis in *D. melanogaster* males. The X-chromosome of *D. melanogaster* contains repeats of genes *Stellate* that encode a protein homologous to the regulatory subunit of protein kinase CKII [122-124]. In the absence of the Y-chromosome the level of transcription of *Stellate* repeats sharply increases that results in a disorder in meiosis and partial or full sterility of males [125]. In the Y-chromosome Su(Ste) (Suppressor of *Stellate*) repeats are located which are highly homologous to *Stellate* genes [126]. Removal of a small region of the Y-chromosome that contains these repeats results in hyperexpression of *Stellate* genes. In addition to the region homologous to *Stellate*, each Su(Ste) repeat carries the insertion of an imperfect transposon *hoppel* which has promotors of anti-sense Su(Ste) RNA located inside [126]. The production of the anti-sense Su(Ste) RNA was detected by RT-PCR [127]. The presence of both sense and anti-sense transcripts suggests the possibility of production of double-stranded Su(Ste) RNA which due to high homology with *Stellate* can cause degradation of *Stellate* mRNA by RNA interference mechanism. This conclusion is supported by detection of siRNA of 25-27 b.p. size homologous to the sequences of *Stellate* and Su(Ste). To suppress *Stellate*, products of *aubergine* and *spindle-E* genes are required: mutants by these genes display no suppression [127-129]. The aubergine and spindle-E proteins belong to the PPD and ATP-dependent RNA helicase families, respectively. Proteins of these families are absolutely necessary for realization of RNA interference, and this also confirms its involvement in the suppression of *Stellate* genes (for details see [130, 131]).

RNA is a surprising substance that strikes by variety of its types and functions and by the beauty and coordination of processes with its involvement [132]. Two decades ago only three types of functionally different RNA molecules were known: ribosomal (rRNA), transfer (tRNA), and messenger RNA (mRNA). Since that time the situation has fundamentally changed. In addition to rRNA and tRNA, hundreds of other RNA types have been found which do not encode proteins but are responsible for regulatory and catalytic functions. Thus, Xist- and roX-RNA were recently shown to be involved in inactivation of the X-chromosome in mammals and in dose compensation of the X-chromosome in *D. melanogaster*, respectively [133]; tmRNA is responsible for targeting and degradation of incompletely synthesized proteins in bacteria [134]; various snRNA are involved in splicing and maturation of RNA-transcripts in eucaryotes [135]; lin-4 and let-7 stRNA play an important role in the regulation of gene expression during development [104, 136]; micro-RNA very similar to stRNA is likely to play an important role in the regulation of expression [116]; besides, small nucleolar RNA is involved in site-specific modifications of rRNA [137, 138]; and some RNA types in mitochondria of protozoa are involved in edition of the genetic information contained in mRNA [139].

The discovery of RNA interference brings up the question of intracellular functions of double-stranded RNA and of the nature and functions of siRNA [3, 114, 140-142]. For today, there is no adequate answer to these questions and to the question about the mechanism of RNA interference. But experimental data available suggest rather definitely that RNA interference is very significant for the cell defense against heterologous genetic information and virus infection. RNA interference is involved in the regulation of mobility of transposons and maintaining of the genome stability, and it is also an important mechanism responsible for the control of gene expression and regulation of development.

The functions of siRNA are not limited to the contribution to RNA interference. The role of siRNA has been recently shown in the regulation of DNA rearrangement in the macronucleus of *Tetrahymena thermophila* [143, 144]. siRNA and proteins required for RNA interference, such as ago1 (an analog of Argonaute), dcr (a protein of the RNAse III family, an analog of Dicer), and rdp1 (a homolog of the RNA-directed RNA polymerase), are involved in production of inactive chromatin in the region of the centromere and in the mat2/3 locus of the yeast *Schizosaccharomyces pombe* [145-147].

RNA interference is extremely significant as an approach for production of knockout organisms and cell lines. The genome of various organisms including the human genome is already sequenced. By computerized analysis of genome sequences the so-called open reading frames, or supposed genes with unknown functions, were detected. The production of knockouts of these genes and screening of resulting phenotypes is a promising approach to elucidate functions of these regions of DNA. Without under estimating of the role and importance of convenient methods for production of knockouts, RNA interference provides for some important advantages.

1. Ease and simplicity combined with high efficiency and specificity.

2. The possibility to concurrently suppress several genes.

3. The introduction into organisms of vectors expressing double-stranded RNA promotes suppression of the gene expression in a certain tissue or at the certain stage of development and also produces knockouts of genes whose absence on the early stages of embryogenesis is lethal.

4. RNA interference can be used to create libraries of organisms and cells with certain genes cut out. Such a library based on *E. coli* clones already exists for *C. elegans*. Findings in functional genomics obtained on its basis are impressive. The elaboration of similar systems for mam-

malian cells is a problem for the future. It is difficult to say how effective these systems should be to realize programs of functional genomics of mammals including humans. However, experimental data on RNA interference in mammalian cells are promising.

At present, we are at the beginning of the RNA interference era in molecular biology. One can hope that RNA interference will be soon a routine laboratory procedure, comparable to the PCR method.

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