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REVIEW

Heterochromatin Formation: Role of Short RNAs and DNA Methylation[1]

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Abstract—The role of small double-stranded RNAs is considered in formation of silent chromatin structure. Small RNAs are implicated in the regulation of individual gene transcription, suppression of transposon expression, and in maintaining functional structure of extended heterochromatic regions. Interrelations between short RNA-dependent gene silencing, histone modifications, and DNA methylation are discussed. Specific features of RNA-induced chromatin repression in various eucaryotes are also described.

Key words: heterochromatin, RNA interference, double-stranded RNA, short RNAs, gene silencing, DNA methylation, transposons

Modifications of histones inside nucleosomes are a common mechanism of regulation of chromatin structure and gene transcription in eucaryotes. The variety of possible modifications of histones and their combinations creates the so-called histone code, which is a series of epigenetic labels specifically recognizable by activating or repressing factors [1]. Thus, methylation of a lysine (K) in histone H3 at position 9 from the N-terminus of the molecule ($H3K9^{Me}$) is an important modification specific for inactive chromatin [2-5]. On the contrary, in chromatin of actively transcribed genes histone H3 has, as a rule, acetylated lysine 9 ($H3K9^{Ac}$) and also methylated K4 ($H3K4^{Me}$). These and many other histone modifications have similar functions in gene repression or activation in yeast, the nematode *C. elegans*, *Drosophila*, mammals, and plants [5-9]. Along with $H3K9^{Me}$, inactive chromatin can be associated in plants, some fungi, and vertebrates with the presence of 5-methylcytosine in DNA that ensures an additional and deeper repression. However, DNA methylation is a less conservative pathway in evolution than the histone code and is absent (or virtually absent) in yeasts, nematodes, and *Drosophila* [10-12].

Genomes of eucaryotes contain extended regions of actively transcribed euchromatin and silent compacted heterochromatin, which is subdivided into constitutive and facultative heterochromatin [13]. Constitutive heterochromatin is mainly located in telomeric and centromeric regions of chromosomes and is filled with repeated sequences and insertions of mobile elements. The number of unique genes in constitutive heterochromatin is extremely low. Inactivation of one of two X-chromosomes in mammals exemplifies generation of facultative heterochromatin [14]. Heterochromatin is characterized by hypoacetylated histones and a high content of $H3K9^{Me}$, and heterochromatic regions in plants and mammals usually contain methylated DNA [1, 10, 15]. Methylation of histone H3 is necessary for binding conservative proteins of heterochromatin, in particular, heterochromatic protein 1 (HP1) [16, 17]. Although pathways of heterochromatin formation are been intensively studied, the initiation stage of this process is still obscure. At present, special noncoding RNA molecules are thought to play a significant role in induction of heterochromatin formation [14, 18, 19]. In particular, mechanism of chromatin repression through double-stranded

Abbreviations: dsRNA) double-stranded RNA; RNAi) RNA interference; RISC) RNA-induced silencing complex; siRNA) short interfering RNA; RdRp) RNA-directed RNA polymerase; HMT) histone methyltransferase; HDAC) histone deacetylase; RITS) RNA-induced initiation of transcriptional silencing; MBD) methyl DNA binding domain; LTR) long terminal repeats.

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[1] This paper was written in honor of the anniversary of B. F. Vanyushin (see special issue of *Biochemistry* (Moscow) (2005) **70**, No. 5), a pioneer in studies on DNA methylation. In the 1960-1970s, he found variations in DNA methylation during development and also association of this process with tumorigenesis.

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RNA (dsRNA) and so-called "short RNAs" is an example of involvement of RNA molecules in epigenetic regulation.

MECHANISMS OF GENE SILENCING THROUGH dsRNA

In 1998, dsRNA molecules injected into the nematode *C. elegans* were found to effectively repress expression of genes homologous in nucleotide sequence (the phenomenon of RNA interference (RNAi)) [20]. Afterwards, the same effects of dsRNA were shown in other animals and also in plants, fungi, and protozoa [21-23]. The molecular mechanism of RNA interference investigated mainly in cell extracts from *Drosophila* [24-28] and later on mammalian cell cultures [29-31] may be presented as follows. Long molecules of dsRNA are cut (processed) by nucleases of the RNase III Dicer family into short double-stranded RNAs of 20-25 nucleotides in length (short interfering RNAs (siRNAs)) that have single-stranded protruding dinucleotide 3′-OH ends and phosphorylated 5′-ends [27, 28]. Then the siRNAs can be incorporated into a protein RNA-induced silencing complex (RISC), which "selects" one of the complementary RNA strands [32] and provides its interaction with the homologous mRNA. RISC cuts molecules of the target mRNA in the regions fully complementary to the small RNAs [25, 26, 28], which results in degradation of mRNA. If siRNAs have some nucleotides unpaired with mRNA, mRNA is not cut but its translation on the ribosome is arrested [33, 34]. The reason for repression of translation directed by small RNAs is unclear. The central component of the RISC complex is formed by proteins of the Argonaute (AGO) family [35-37] with characteristic domains PAZ and PIWI [38]. The PAZ domain specifically binds siRNA [39-41], and the PIWI domain similar in structure to RNase H domain [42] can cleave mRNA, as has been shown for the human AGO-2 protein [37]. Conservative proteins of the Argonaute family are found in archaea and in virtually all eucaryotes except the budding yeast *S. cerevisiae*. Mutations in the genes encoding Argonaute proteins disturb dsRNA-dependent silencing in eucaryotes [43-50].

Small RNAs of other origins, the so-called microRNAs, can also repress gene expression. These RNAs are produced by the nuclease Dicer from hairpin RNA precursors encoded in genomes of animals and plants [51]. Conversely to the long molecule of dsRNA that is cut by Dicer into a statistical set of siRNAs different in nucleotide sequence, microRNA is always an oligonucleotide cut from a hairpin-like precursor at strictly definite positions. MicroRNAs are involved in regulation of different mRNA expression, usually via binding to complementary sites in 3′-non-translated regions. Similarly to siRNAs, microRNAs cut a target mRNA in the case of perfect complementarity of their nucleotide sequences or stop the translation if their complementarity is partial [51, 52].

In addition to the above-described post-translational repression of mRNA in the cytoplasm, siRNAs can repress transcription of the homologous gene and cause a local change in the chromatin structure in the nucleus. Artificial expression of small RNAs corresponding to an actively transcribed gene represses the transcription in fission yeast *S. pombe*, plants, and human cells, which is correlated with an increase in the amount of methylated histones $H3K9^{Me}$ [53-55]. In mammals and plants, the repression induced by siRNAs was also accompanied by methylation of homologous regions of DNA [55-57]. Thus, molecules of siRNA produced from the long dsRNA can induce a local formation of repressive chromatin. No direct induction of transcriptional repression by homologous dsRNA was detected in the nematode and *Drosophila*. Nevertheless, some data suggest a possible influence of *Drosophila* RNAi machinery on the chromatin structure [58]. The involvement of endogenous dsRNA molecules in formation of heterochromatin was shown for *S. pombe* and plants [48, 54].

Thus, different effector complexes containing small RNAs can repress translation, cleave mRNA (the RISC complex), or induce chromatin repression (the RITS complex, or RNA-induced initiation of transcriptional silencing complex). These multiprotein complexes include different sets of proteins, but they all include proteins of the Argonaute family [59-61]. Mutations in the Argonaute-encoding genes affect the dsRNA-dependent degradation of mRNA [35, 37, 43, 45, 59, 60], translational repression through microRNAs [51, 59, 62], and also the transcriptional repression caused by small RNAs [44, 48, 54].

We shall consider mechanisms of the dsRNA-dependent heterochromatin formation in various eucaryotes and its role in regulation of the organism's inherent genes, defense of the genome against foreign DNA sequences (transgenes), and repression of activities of mobile elements.

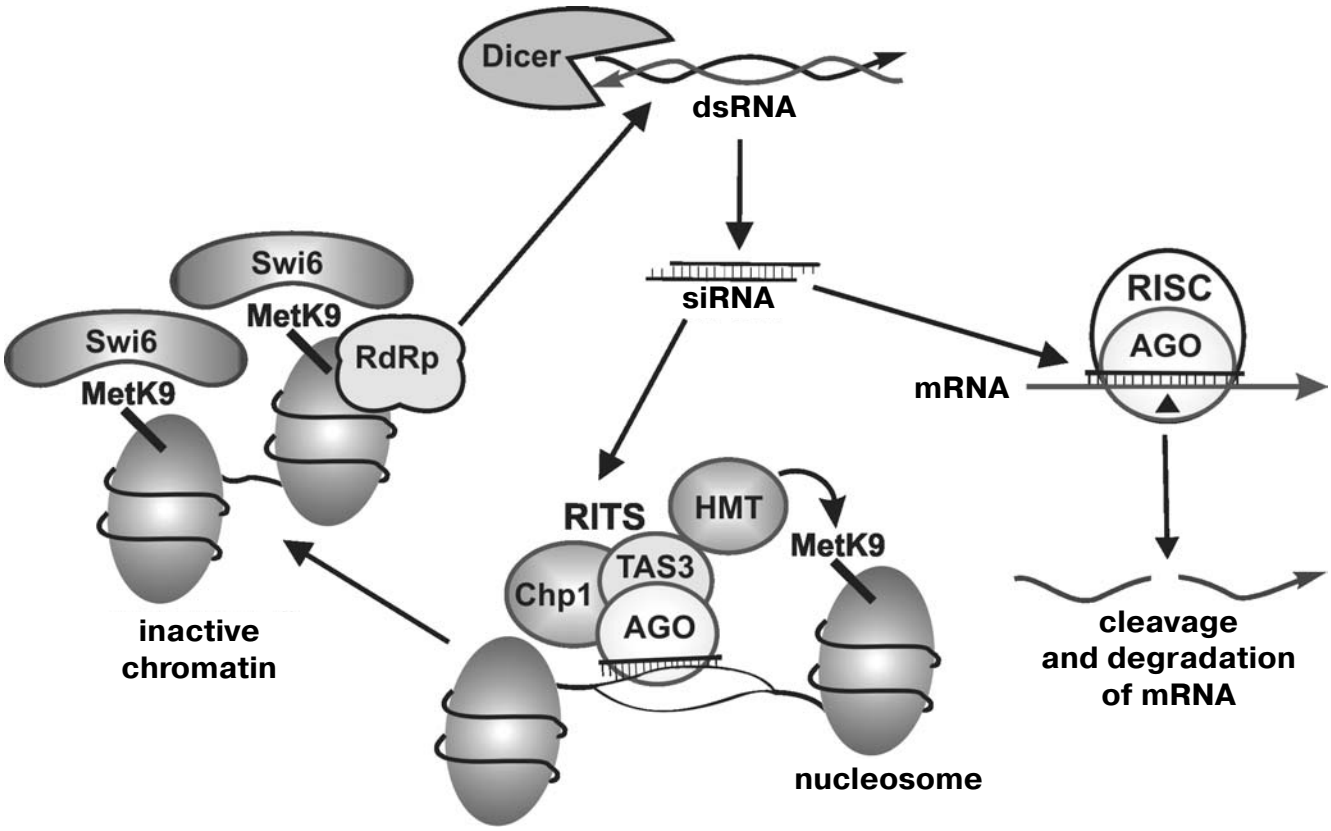
SMALL RNAs AND FORMATION OF HETEROCHROMATIN IN YEAST

Involvement of siRNAs in histone modification was shown in studies on heterochromatin of centromeric regions of chromosomes in the fission yeast *S. pombe* [48]. Chromatin of the centromere region is enriched with $H3K9^{Me}$ and the Swi6 protein, which is a yeast analog of the HP1 protein of multicellular eucaryotes [63]. Repeated sequences in the centromere region similar in structure to mobile elements are poorly transcribed. The transcription occurs from both strands of DNA, which results in double-stranded transcripts. dsRNA can also be

produced on the template of single-stranded RNA with involvement of RNA-directed RNA polymerase (RdRp), which has been detected in the centromere heterochromatin [48]. Using an approach of "short RNA cloning", siRNAs were found with sequences corresponding to those of centromere repeats [64]. Mutations in the genes encoding Dicer, the Argonaute protein, and RdRp resulted in the loss of $H3K9^{Me}$ and Swi6 in the centromere heterochromatin and active transcription of the repeats [48]. A similar effect is caused by mutations in the genes encoding Swi6 and histone methyltransferase (HMT) methylating K9 of the H3 histone. According to the model presented in Scheme 1, the dsRNA produced on the centromere repeats is cut by nuclease Dicer into siRNAs, which in the protein complex RITS interact with the homologous centromere DNA regions and recruit HMT [48]. The protein Swi6 binds to $H3K9^{Me}$, and this leads to chromatin compactization.

The RITS complex responsible for chromatin repression was found to include the following proteins: Argonaute, TAS3 with an unknown function, and Chp1 [61]. Chp1 contains a conservative chromodomain specific for proteins of chromatin and seems to be responsible for stabilization of RITS on centromeric repeats. It remains unclear how siRNAs in the RITS complex can recognize their targets. Possibly, siRNAs interact with homologous DNA forming a RNA–DNA hybrid, or siRNAs bind to nascent transcripts, and the RITS complex acquires the ability for attracting histone methylase to the adjacent region of chromatin.

RdRp seems to be responsible for increasing the silencing. Two possible mechanisms are supposed that would allow RdRp to amplify centromeric transcripts but not other RNAs of the cell. First, RdRp can use single-stranded siRNA as a primer for synthesis. Second, RdRp detected in the complex with repressed chromatin can amplify *in situ* RNA transcribed from centromeric repeats [48, 65, 66]. The described scheme of silencing of centromeric repeats is modeled by introduction into the genome of *S. pombe* of a construct producing dsRNA



Mechanism of repression of the gene expression in *S. pombe* with involvement of dsRNA. Long dsRNA molecules produced as a result of bidirectional transcription or by RNA amplification with involvement of RNA-directed RNA polymerase (RdRp) are cut by endonuclease Dicer into siRNAs. siRNAs inside the protein complex RISC cleave complementary molecules of mRNA, and inside the RITS complex they interact with the genomic locus homologous in nucleotide sequence. The RITS complex, which includes the proteins Argonaute (AGO), TAS3, and Chp1 recruits histone methylase (HMT), which methylates K9 of histone 3 (MetK9). The protein Swi6 binds to MetK9, resulting in chromatin compactization. RdRp interacts with the repressed chromatin and provides synthesis of dsRNA on the template of a newly produced single-stranded RNA and thus enhances the repression

Scheme 1

homologous to the selected gene, which leads to H3K9 methylation, binding of Swi6, and inactivation of gene transcription [53].

The presence of Swi6 on centromeric repeats is necessary for binding of cohesin, a conservative protein responsible for adequate functioning of the centromere during segregation of chromosomes in mitosis [67]. Mutations affecting production of any component of the silencing system (Argonaute, Dicer, RdRp, and also Swi6 and HMT) block mitosis at the anaphase stage [68, 69]. Thus, small RNAs play an important role in cohesion of chromatides and promotion of normal mitosis in yeast.

The RNAi machinery in *S. pombe* also assures the transcriptional repression of the gene group responsible for meiosis [53] (see below). However, disorders in the genes of RNAi do not disturb the maintenance of the facultative heterochromatin structure in the locus which determines the mating type (MAT locus) but lead to defects in establishment of repression [70]. Note also that the global analysis of regulation of *S. pombe* transcriptome performed with microarrays failed to reveal a pronounced correlation between the HMT-directed repression and RNAi [71]. Thus, in many cases repressed chromatin is maintained without involvement of small RNAs.

SPECIFIC FEATURES OF RNA-SILENCING IN PLANTS

As in the case of *S. pombe*, dsRNA induces in plants formation of a repressed chromatin structure in the genes with complementary sequences. However, in contrast to yeast, the repression is achieved not only via methylation of histones but also via methylation of DNA. This process is described as RNA-dependent DNA methylation. Transgenic constructs producing dsRNA homologous to promoters of the genes cause their methylation and decrease the level of transcription [56, 72]. Mutants in the genes of Dicer, Argonaute, and RdRp are at least partially deficient in DNA methylation (see below) [44, 54, 73-76].

The mechanism of the RNA-dependent DNA methylation in plants is involved in repression of transposons, regulation of some euchromatic genes, and also serves to fight with viruses and leads to repression of transgenes introduced into the plant genome (a cosuppression phenomenon, see below). The RNAi machinery is also required for production and functioning of microRNAs regulating expression of a great variety of plant genes, mainly on the post-transcriptional level [51, 77]. However, some microRNAs of plants induce methylation of complementary sequences of DNA [78].

Note that cognate RNAi proteins in plants are encoded by a number of genes, and this significantly complicates genetic study on silencing. As differentiated from the *S. pombe* genome, which includes unique genes encoding Dicer, RdRp, and Argonaute, *Arabidopsis* has four different genes of Dicer, at least three functionally active RdRp, and ten genes of Argonaute [46, 74]. Different proteins of the same family can be involved in different types of RNA silencing or have overlapping functions. Moreover, some proteins can be expressed tissue-specifically or only during definite stages of development, as shown for AGO7 in *Arabidopsis* [79].

In plants, two classes of siRNAs are found: short ones of 21-22 and longer ones of 24-26 nucleotides in length [80, 81], which in *Arabidopsis* are produced with involvement of the proteins Dicer2 and Dicer3, respectively (Scheme 2) [74]. The short siRNAs are supposed to be implicated in cleavage of RNA molecules in the RISC complex, whereas the "long" siRNAs repress chromatin and methylate DNA [74, 80]. Moreover, the "long" siRNAs can act as signaling molecules in systemic silencing [80, 82], i.e., spreading of the repression induced in a plant local area to the whole organism [77, 83]. Contrasting to plants, the only Dicer in *S. pombe* produces siRNAs, which perform both degradation of mRNA and silencing on the chromatin level [60].

COSUPPRESSION IN PLANTS

The cosuppression phenomenon is a dramatic fall in the total level of expression of transgenes or even its complete inhibition, together with increase in the number of their copies in the genome. If transgenes are homologous to an inherent gene of the organism (endogene), the expression of the latter is also repressed. Cosuppression is found in various plants and also in the fungus *Neurospora crassa* [84-86]. In the latter case, this phenomenon is called quelling. The cosuppression was described in 1990, long before discovery of repressing properties of dsRNA: introduction into the genome of *Petunia hybrida* of additional copies of the gene responsible for synthesis of the red pigment of flowers paradoxically lowered the amount of the pigment as compared to nontransformed plants [87, 88]. Studies on different systems have revealed that cosuppression occurs as a result of both cleavage of mRNA molecules in the cytoplasm (post-transcriptional gene silencing (PTGS)) and suppression of transcription (transcriptional gene silencing (TGS)) caused by methylation of cytosine residues in promoters of transgenes and homologous endogenes [89-92].

Based on detection of complementary to transgenes short RNAs in correlation with the presence of silencing [93], it was suggested that cosuppression should be determined by production of dsRNA. dsRNA can arise as a result of bidirected transcription of transgenes or with involvement of RdRp, which binds to transgenic RNA and thus synthesizes the complementary strand [73, 94]. RdRp can synthesize RNA without a primer, or use single-

stranded siRNA as a primer. In the latter case, the repression can spread towards the 5′-end of the gene if initially only siRNA complementary to the 3′-end of the gene existed [95]. Amplification of RNA with siRNA primer and without it was shown *in vitro* on plant cell-free systems [81]. However, it remains unclear how RdRp, when initiating cosuppression, can recognize RNA which is read from transgenes and differentiate it from other RNAs. Possibly, only those RNA molecules are amplified that are accumulated in an unnatural excess. In fact, introduction of transgenes into the genome under the control of strong promoters results in more pronounced cosuppression [96, 97]. Moreover, aberrant transcripts, e.g., lacking the cap, can be preferential templates for RdRp. Cosuppression is strengthened with mutation in the gene of 5′-3′-exonuclease, which destroys capless mRNAs [98]. In the plants with concurrent mutations in the genes encoding this nuclease and RdRp, capless transcripts are accumulated [98]. But the reason for appearance of aberrant transcripts is unknown. It seems that the stage of incorporation of transgenes into the genome determines the subsequent production of dsRNA. According to this model, the transgene at the moment of integration receives certain "epigenetic labels", e.g., histone modifications or methylated cytosines in DNA, and this results in the interaction of the transgene locus in the genome with RdRp amplifying the locally produced transcripts. Such a mechanism could appear in the course of evolution of plants as an approach for defense against invasion of retroviruses and mobile element transpositions.

Mutations affecting cosuppression of transgenes also lead to supersensitivity of plants to RNA viruses [86]. Silencing of viruses is triggered by dsRNA intermediates produced during viral replication. If transgenes inside the plant genome contain sequences homologous to RNA virus, the succeeding infection of the plant with this virus results in methylation of the transgenic DNA [99-101]. Transgenes containing viral sequences induce cleavage of the homologous viral RNA and prevent viral infection [102-104]. Thus, silencing based on formation of dsRNA allows plants to struggle against exogenous genes, and this is a kind of immune defense on the level of nucleic acids [105].

DNA METHYLATION AND THE RNAi MACHINERY

In plant genomes cytosine residues are methylated in palindromic CG, CNG, and also asymmetric CNN sites (where N is any nucleotide). In fungi and mammals, mainly CG sites are methylated [11, 106]. Two types of DNA methylation have been described: the *de novo* methylation and maintenance of methylation on a "half-methylated" template, which after the replication carries methylated cytosines only in the "parental" strand. The maintenance of methylation provides a stable inheritance of the repressed state of DNA through cell divisions. The genome of *Arabidopsis* contains at least ten genes, which encode DNA methyltransferases, and four of them have been characterized [107]. The methylase MET1 homologous to the Dnmt1 methylase of mammals performs both *de novo* [108] and maintenance of methylation of CG sites [108-110]. The methylases DRM1 and DRM2 (Domains Rearranged Methylase) are required for *de novo* methylation of sites of all types and are also involved in maintenance of methylation of CNG and CNN sites [54, 111, 112]. The chromodomain-possessing chromomethylase3 (CMT3) is involved in methylation of CNG and CNN sites together with DRM1/DRM2 [111, 113, 114]. Mutation in the gene DDM1 (Decrease DNA Methylation 1) encoding a component of the ATP-dependent complex of chromatin remodeling also affects methylation of all type sites of DNA in *Arabidopsis* [115]. This complex changes the spatial arrangement of DNA on the histone globule and replaces the latter along the DNA chain [116]. However, the specific role of the remodeling complex affecting methylation of DNA is unknown.

The interrelation between methylation of histones and DNA was first detected in the fungus *N. crassa*. Mutation in the gene *dim5* encoding the H3K9 histone methyltransferase disturbs methylation of both histones and DNA [117, 118], whereas mutation in the gene of the only DNA methylase dim2 totally abolishing the genome methylation has no effect on methylation of histones [119]. Thus, DNA methylation in *N. crassa* is determined by methylation of K9 of the histone H3, but not conversely. A direct interaction of DNA methyltransferase dim2 with HP1 was shown [120]. Consequently, HP1 in *N. crassa* acts as a "bridge" between $H3K9^{Me}$ and methylation of cytosines in DNA. In plants (*Arabidopsis*), the opposite process is detected: histone methylation is directed by methylated CG sites of DNA [121-123]. But methylation of the sites CNG and CNN in *Arabidopsis* depends on methylation of histones [124, 125].

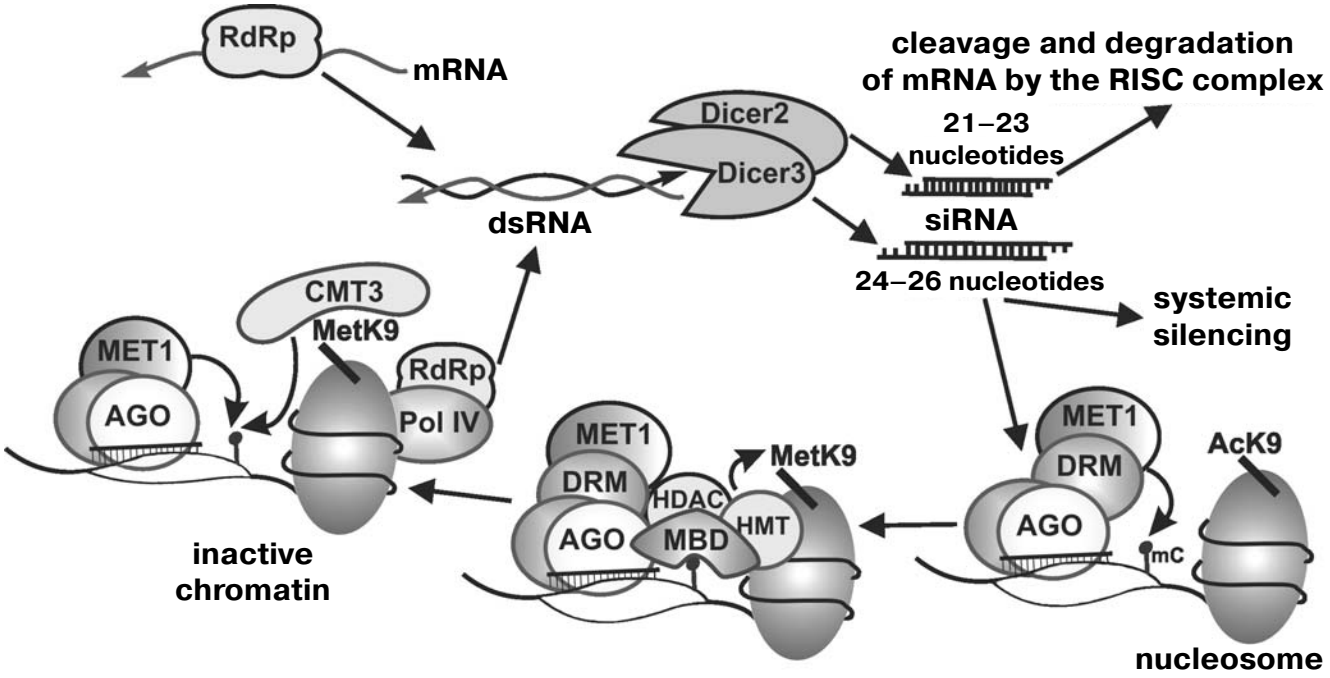
Methylation of DNA sequences caused by homologous dsRNAs was shown in plants and later in mammals. However, methylation not always depends on RNA. The fungus *N. crassa* possesses both RNA silencing and DNA methylation systems, but they are not interrelated [126, 127]. Mutations in the genes of RNAi in *N. crassa* do not decrease the level of the genome methylation, which mainly concerns repeating elements [127]. Note that in plants RNA-independent DNA methylation seems also to be more common than RNA-dependent methylation [128, 129].

In *Arabidopsis* different systems of genes control initiation and maintenance of the RNA-dependent DNA methylation. This was shown for silencing of the FWA gene encoding a homeoprotein that controls flowering. The promoter of FWA contains tandem repeats, which are targets for endogenous siRNAs arising during tran-

scription of mobile elements (see below) [129]. The transgene FWA is repressed when integrated in the genome of the wild type plant but not of mutants in Dicer3, RdRp, AGO4 [75], and also double mutants in the genes encoding DNA methylases of DRM1/DRM2 [75, 111, 112]. However, these mutations have no influence on maintenance of methylation of CG sites and do not cause derepression of endogenous FWA [75]. Thus, the proteins responsible for formation and functioning of siRNAs are involved only at the stage of establishing methylation. The repressed chromatin in the following generations is maintained with involvement of DNA of the MET1 methylase. Mutation in MET1 results in derepression of endogenous FWA because of demethylation of the CG sites [130].

Genetic studies on different systems of silencing allow us to illustrate the mechanism of the RNA-dependent DNA methylation in plants with the following Scheme 2. Long molecules of dsRNAs produced by replication of an RNA virus in the cytoplasm, action of the cellular RdRp, or via bidirectional transcription of transgenic or endogenous DNA are cut by the protein Dicer3 into siRNAs of 24-26 nucleotides in length. These siRNAs are included into the complex, which seems to be like the RITS complex in yeast and contains a protein of the Argonaute family. This complex interacts with chromatin and recruits methylases capable of *de novo* methylating DNA: DRM1/DRM2 modify the CNG/CNN sites and three methylases (MET1/DRM1/DRM2) methylate the CG sites [75, 108, 111]. The ATP-dependent complex of nucleosome remodeling [131], which provides the availability of certain DNA regions for methylation, can also be involved. Methylated cytosines are recognized by proteins containing methyl DNA binding domains (MBD), which can bind histone deacetylases (HDAC) and methyltransferases (HMT), which are involved in RNA-dependent methylation [54, 132]. HDAC removes the acetyl group from K9 of histone H3, opens lysine 9, and makes it available for HMT. $H3K9^{Me}$ is recognized by the chromodomain of the DNA methylase CMT3, which together with DRM1/DRM2 maintains methylation of the sites CNN and CNG, whereas MET1 maintains methylation of the CG sites.

Mutations in the genes encoding the DNA methylase MET1 and protein DDM1 lead to disappearance of



Mechanism of chromatin repression directed by dsRNA molecules in plants (*Arabidopsis*). Molecules of dsRNA are produced as a result of RNA replication or transcription of some loci in the genome. The Dicer proteins process dsRNAs with formation of two types of siRNAs: short ones of 21-22 nucleotides and more extended ones (of 24-26 nucleotides). siRNAs of 21-22 nt in length are involved in cleavage of RNA molecules; siRNAs of 24-26 nt in length perform repression on the chromatin level and are also involved in the systemic spreading of silencing in the organism. The complex interacting with chromatin and containing siRNA and AGO attracts methylases DRM and MET1 which *de novo* methylate DNA. Methylated cytosines (mC) are recognized by the MBD proteins, which can interact with histone deacetylases (HDAC) and methyltransferases (HMT). Histone deacetylase removes the acetyl group of lysine 9 of histone H3 (AcK9). Histone methylase methylates lysine 9 (MetK9), which is recognized by the chromodomain of DNA methylase CMT3. Methylation is maintained with involvement of DNA methylase MET1. DNA in the repressed chromatin is transcribed by RNA polymerase IV (Pol IV), whereas newly synthesized transcripts are used as a template for the synthesis of dsRNAs by RdRp

Scheme 2

siRNAs in various systems of silencing that suggests the involvement of these proteins in stabilization of siRNAs [128, 129]. To confirm this model, additional biochemical studies are necessary. One may speculate that the production of siRNAs is not only the cause but also the consequence of DNA methylation. That means that the repressed structure of chromatin of the locus can somehow stimulate a local production of small RNAs.

One *Arabidopsis* gene identified during the search for suppressors of RNA silencing was found to encode a protein homologous to the large subunit of DNA-dependent RNA polymerases [133, 134]. This protein carries a unique C-terminal domain but contains the most conservative regions of the RNA polymerase subunit. Therefore, plants were suggested to have RNA polymerase Pol IV different from the three RNA polymerases. The genome of *Arabidopsis* was found to contain genes that seemed to encode other subunits of Pol IV [133, 134]. The knockdown of these genes through homologous dsRNA resulted in the same disorders in RNA silencing as mutation in the gene of the large subunit, in particular, disappearance of some endogenous siRNAs and disorders in the RNA-dependent DNA methylation. Genetic approaches revealed Pol IV to be involved in silencing in *Arabidopsis*, together with Dicer 3 producing "long" siRNAs of 24-26 nucleotides in length, and one RdRp (RDR2). It was suggested that, by contrast with RNA polymerases I-III, which preferentially transcribe genes inside the "open" chromatin, Pol IV should have an increased affinity for compacted chromatin carrying $H3K9^{Me}$ and methylated DNA. Interacting with Pol IV and using the synthesized transcripts as a template, RdRp can produce dsRNA corresponding to the regions of heterochromatin (Scheme 2). Thus, Pol IV/RdRp can be responsible for provision of the pool of siRNAs maintaining the repressed state of chromatin [133, 134]. As it has been said, the interaction of RdRp with chromatin may be an explanation of arising of dsRNAs during cosuppression.

ROLE OF dsRNAs IN TRANSCRIPTIONAL REPRESSION OF MOBILE ELEMENTS IN PLANTS AND YEAST

Various eucaryotes have dsRNAs and siRNAs corresponding to sequences of retrotransposons and DNA-transposons [74, 135-140]. Mobile elements can often be transcribed both forwards and backwards. The opposite direction of transcription can depend on the own "antisense" promoters of transposons or external promoters close to insertion of the element [141]. In addition, inverted regions in transcripts of mobile elements can produce hairpin-like structures [138]. dsRNAs can also originate from heterochromatin where abundant transposable elements with mutual opposite orientation are accumulated [129]. Due to the presence of dsRNA transposons, the cellular RNAi machinery can repress their expression, which is often deleterious for the organism because it leads to transpositions and, as a result, mutations and chromosome rearrangements [86, 142]. Some mutations affecting RNAi are also accompanied by derepression of mobile elements in animals [43, 143-148], plants [54, 74], and microorganisms [50, 149].

In *Arabidopsis*, insertions of transposons, often integrated one into another [129], are a significant part of heterochromatin that is located in centromeric and telomeric regions of chromosomes. Moreover, in plant chromosomes heterochromatin forms small easily stainable intercalary areas, or knobs. About 90% of cloned endogenous siRNAs in *Arabidopsis* correspond to sequences of transposons and heterochromatic regions [74, 136]. Such siRNAs have 24-26 nucleotides in length and belong to the "long" siRNAs, which as it has been mentioned are involved in repression of gene expression on the level of transcription. The number of siRNAs corresponding to certain regions of intercalary heterochromatin positively correlates with the methylation degree of both DNA and histones (the presence of $H3K9^{Me}$) in these regions [129]. Mutation in the DDM1 gene results in disappearance of siRNA of intercalary heterochromatin, lowering the number of methylated DNA cytosines and $H3K9^{Me}$ histones, and increase in the amount of the $H3K4^{Me}$ modification that is specific for active chromatin [129].

Silencing of transposons on the chromatin level is caused not only by RNAi but also due to RNAi independent DNA methylation. In *Arabidopsis*, mutations in the genes encoding the Argonaute proteins result in derepression and abolishing of methylation only of some mobile elements [54, 74, 128]. Caused by Dicer 3 or RdRp deficiency, absence of siRNAs corresponding to transposon sequences is not always accompanied by their activation [74]. Mutations in the genes of chromatin proteins (DDM1, MET1) lead to greater derepression of transposons than mutation in the RNAi genes [128, 129, 150, 151].

The mechanism of RNAi directed to repress transposon expression can also regulate transcription of the organism's inherent genes. This occurs in the cases when the transposon repressed with involvement of siRNA is located in the regulatory region of the gene or nearby. Thus, in *S. pombe*, mutations in the Argonaute, Dicer, and RdRp result in activation of a group of genes responsible for meiosis during starvation but repressed during the growth on rich nutrition medium [53]. Insertions of LTR retrotransposons were found near each gene of this group. Endogenous siRNAs corresponding to the LTR sequences were detected. It was suggested that siRNAs should induce methylation of K9 of the H3 histones in the LTR region (similarly to events in the centromeric region), and then the repressed structure of chromatin

spreads from LTR to the adjacent gene with involvement of RdRp. LTR deletions result in histone demethylation and activation of neighboring genes [53]. Thus, retrotransposons in *S. pombe* act as controlling elements of the genome. Involvement of small RNAs of retrotransposons in regulation of transcription of unique proteins encoding genes is also shown for *Arabidopsis*. The above-mentioned gene *FWA* contains in the promoter tandem repeats of a SINE-like retro-element. A homeoprotein encoded by the *FWA* gene is expressed only in the endosperm and is involved in regulation of flowering [130, 152]. In vegetative tissues, siRNAs homologous to the SINE element direct histone and DNA methylation in the promoter of *FWA* [129].

In addition to mobile elements, inverted repeats of some genes can also be a source of endogenous dsRNA [54, 153]. Thus, the euchromatin gene *PAI*, which controls biosynthesis of tryptophan in *Arabidopsis*, is expressed poorly because of transcription of inverted repeats of two other copies of this gene and production of dsRNAs [153, 154]. Methylation of *PAI* is prevented when transcription of inverted repeats is disturbed [153].

RNA SILENCING ON THE CHROMATIN LEVEL IN ANIMALS

In *Drosophila*, DNA is methylated much more weakly than in plants and mammals [12, 155, 156], and the functional role of this methylation is unclear. Therefore, *Drosophila* is interesting as a multicellular organism in which the repressed state of chromatin, including heterochromatin, can be established and maintained without methylation of DNA observed in unicellular *S. pombe*. Gene knockdown with artificially synthesized dsRNAs or by introduction of dsRNA-producing constructs into the genome is an approach intensively used in functional genomics of *Drosophila* [157, 158]. But induction of transcriptional repression of active euchromatic genes by "exogenous" dsRNAs was not shown in *Drosophila*. However, disorders in RNAi proteins in *Drosophila* were found to change the state of heterochromatin [58]. Endogenous small RNAs are supposed to repress chromatin via negative modifications of histones, recruitment of the heterochromatic proteins HP1 and HP2, and, possibly, transcriptional repressors presented by proteins of the Polycomb group [58, 159]. Mutations in *Drosophila*'s genes of RNAi that encode proteins of the Argonaute family and RNA helicase abolish the position effect [58], which presents an unstable inactivation of the gene adjacent to heterochromatin. The same mutations affect the normal distribution of the HP1 and HP2 proteins on chromosomes and decrease the total amount of $H3K9^{Me}$ histones [58].

The RNAi mechanism is involved in repression of retrotransposons in germinal tissues of *Drosophila* [145, 147, 148]. Activation of some retrotransposons caused by mutation in the RNAi gene of is accompanied by a local decondensation of chromatin and increase in the histone acetylation (Klenov, unpublished data). It seems that small RNA can recruit histone deacetylase to the repressed region of chromatin, similarly to that observed in plants. In germinal tissues of *Drosophila* males, dsRNA produced by heterochromatic repeats *Su(Ste)* directs silencing of the homologous genes *Stellate* whose overexpression disturbs normal spermatogenesis [22, 145, 160]. Derepression of the *Stellate* genes caused by deletion of the *Su(Ste)* locus or mutation in the genes of RNAi is accompanied by disappearance of small RNAs [145, 161]. The locus *Su(Ste)* deletion is associated with accumulation of unspliced *Stellate* transcripts, suggesting an increase in the level of *Stellate* transcription because the splicing goes cotranscriptionally in the cell nuclei. The *Su(Ste)* deletion increases derepression of the *Stellate* gene cluster in euchromatin several tens times higher than of the *Stellate* genes located in heterochromatin (Klenov, unpublished data). These data suggest that small RNAs can induce transcriptional repression of genes in germinal tissues of *Drosophila*.

Transcription of the *Stellate/Su(Ste)* genes and mobile elements seems to be repressed through "long" siRNAs of 24-26 nucleotides in length, similarly to events in plants [139, 140, 161], whereas siRNAs of 20-22 nucleotides in length are involved in cleavage of mRNA or repression of translation [28, 139]. The cloning of small RNAs of *Drosophila* has also revealed two classes of small RNAs with different length: microRNAs of 20-22 nucleotides and small RNAs corresponding to transposons, which as a rule have more than 24 nucleotides in length [139].

Repression of chromatin in mammals was recently shown to be induced by the RNAi. Introduction of artificially synthesized siRNAs into human cell culture resulted in methylation of CG sites of DNA, methylation of K9 of histone H3, and transcriptional repression of the homologous gene [55, 57]. It remains unclear whether endogenous molecules of dsRNAs are involved in formation of heterochromatin in mammals. Knockout of the gene encoding Dicer in the culture of hybrid chicken/HeLa cells results in accumulation of transcripts of the α-satellite repeats that form centromeric regions of human chromosomes and also disturbs the sister chromatide segregation during mitosis [162]. This seems to be similar to the mechanism of maintaining of the centromere heterochromatin functions in yeast. The null-mutation in the Dicer gene in mice results in an early arrest of development associated with deficient stem cells [163, 164]. However, this effect can be caused by disorders in the structure of centromeric chromatin and normal division as well as by disappearance of microRNAs regulating the expression of different mRNAs during the mouse development.

dsRNA-DEPENDENT HETEROCHROMATINIZATION AS AN ACQUIRED FUNCTION OF GENE REPRESSION IN EUCARYOTES

Various cases of gene silencing with involvement of small RNAs suggest that this mechanism appeared in eucaryotes at early stages of evolution to protect the genome against expansion of mobile elements and viruses. From the evolutionary standpoint, it is an interesting possibility of "taming/domestication" of mobile elements and using the mechanism of their transcriptional silencing to support cellular functions of the host organism. Such a tendency is exemplified by maintenance in yeast of the functional state of the centromeric regions consisting of transposon-like repeats and also by cases of repression of genes with regulatory regions containing retrotransposons or their fragments. In *S. pombe*, dsRNA molecules corresponding to transposon sequences regulate transcription of the genes responsible for transition from mitotic divisions to meiosis and sporogenesis [53]. Thus, small RNAs can direct the repression of nonhomologous genes located near long terminal repeats of retro-elements via controlling differentiation of the yeast cell. Small RNAs corresponding to retrotransposons function also in plants and ensure repression in somatic tissues of the *FWA* gene, which controls flowering in *Arabidopsis* [129]. Genes responsible for RNAi and silencing of retrotransposons in *Drosophila* [145, 147] are also involved in development of germinal tissues and gametogenesis [165-168], and this suggests a possible function of dsRNAs of mobile elements in these processes.

The mechanism of chromatin repression through small RNAs in *S. pombe* is characterized by local methylation of K9 of histone H3. In contrast to yeast, small RNAs in plants seem, first of all, to direct methylation of DNA, which stimulates the subsequent methylation of histones. In both plants and yeast, an important role during repression belongs to RNA-directed RNA polymerase, which functions in the cell nuclei and provides formation of dsRNA corresponding to heterochromatin sequences. However, RdRp was not detected (by sequencing) in genomes of *Drosophila* and mammals. Consequently, in these organisms endogenous small RNAs can be produced only as a result of bidirectional transcription or processing of hairpin-like regions of RNA. It was suggested that in the nematode *C. elegans*, dsRNAs did not affect compactization of chromatin. The RNAi machinery in *C. elegans* is involved in repression of tranposons, but this repression likely occurs on the post-transcriptional level and not as a result of chromatin repression [138]. This finding is in agreement with the lack of "classical" chromatin in the nematode.

The involvement of siRNAs in regulation of gene expression shows that not only proteins but also RNA molecules not encoding proteins play roles in this process. MicroRNAs (their regulatory role was beyond consideration here) and siRNAs are only a small fraction of noncoding regulatory RNAs. In eucaryotes, the bulk of DNA is transcribed but not involved in encoding proteins. Note that overlapping transcripts, which are read from both DNA strands, form an essential part of the newly produced RNA in mammals [169]. In addition to heterochromatinization directed by siRNA molecules, other cases of involvement of noncoding RNAs in the regulation of chromatin structure have been described. Thus, noncoding and rather large RNAs (of more than 10,000 nucleotides) regulate activities of the X-chromosome genes on the chromatin level in mammals and *Drosophila* [14]. Inactivation of one X-chromosome in mammalian females through noncoding RNAs is accompanied by methylation of DNA. The components, which are destroyed by treatment with RNases, are also involved in the organization of the centromeric heterochromatin on autosomes in mammals and binding of the repressor protein HP1 to the K9-methylated H3 histones [170].

Note in conclusion that production of short dsRNAs accompanied by chromatin inactivation and its spreading onto adjacent regions allows us to discuss from new viewpoints not only mechanisms of gene repression, but also arising of new functions of heterochromatin and its evolution.

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