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REVIEW

Structure of Telomeric Chromatin in *Drosophila*

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Abstract—The telomeric nucleoprotein complex protects linear chromosome ends from degradation. In contrast to most eukaryotes in which telomerase is responsible for telomere elongation by adding short DNA repeats synthesized using an RNA template, the telomere elongation in *Drosophila* involves transposition of specialized telomeric retroelements onto chromosome ends. Proteins that bind telomeric and subtelomeric sequences form specific telomeric chromatin, and its components are highly conserved among organisms employing different mechanisms of telomere elongation. This review is focused on the analysis of components of the *Drosophila* telomeric complex and its comparison with telomeric proteins in telomerase-encoded organisms. Structural and functional analysis of *Drosophila* telomeres suggests that there are three distinct chromatin regions: protective structure at the very end of chromosome (cap), subtelomeric region which is characterized by condensed chromatin structure, and the terminal retrotransposon array whose expression is under the control of an RNAi (RNA interference)-based mechanism. The link between RNAi and telomeric chromatin formation in germinal tissues is discussed.

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Telomeres are nucleoprotein complexes positioned at the ends of linear chromosomes. The problem of incomplete end replication of DNA was originally raised by the Russian scientist A. Olovnikov [1]. The point is that DNA polymerase cannot fully replicate the end sequences and thus a linear chromosome shortens after each replication cycle; this would result in degradation of subtelomeric genes. One function of telomeric DNA elongation is the compensation of end degradation. In most organisms the enzyme telomerase catalyzes telomere elongation by synthesizing short DNA stretches of 6-11 nucleotides using a RNA template encoded by its own cell genome [2-5]. *Drosophila* telomeres are formed as the result of retrotranspositions of specialized telomeric retrotransposons [6-9]. In such organisms as *Bombyx mori* and *Chlorella* there is a mixed type of telomere elongation: the telomeric retrotransposons are inserted into a sequences of short repeats formed by telomerase [10-12]. In all cases, telomere elongation involves mechanism of reverse transcription, i.e. synthesis of DNA of an RNA template. This gives background for discussion on common origin of telomerase and retrotransposon modes of telomere elongation [13]. Indeed, the reverse transcriptase domain of telomerase shares similarity with the corresponding domain of some retrotransposons [14]. Irrespectively of the nature of telomeric sequences, they all have a buffer function. The protein complex specifically recognizing telomeric DNA participates in regulation of telomere length; this is the most important factor for normal development of the cell. Besides DNA end underreplication, the ends may undergo repair processes (as in the case of double-stranded DNA breaks); this may cause telomere fusion and impairments of chromosome integrity. This unwanted process is prevented by formation of a cap, which is comprised of a protein complex at the chromosome end. Telomeres together with centromeres also play a very important role in mitotic and meiotic chromosome behavior; in the interphase nucleus, they determine a "chromosomal territory" of each chromosome in the nuclear space. These processes now attract much attention in relation to the recognized role

Abbreviations: ATM kinase) ataxia-telangiectasia mutated kinase, the kinase defective in the ataxia-telangiectasia syndrome; dsRNA) double stranded RNA; HOAP) HP1/ORC-associated protein, the protein interacting with HP1 and ORC (origin recognition complex); HP1) heterochromatic protein 1; miRNA) microRNA; PcG) Polycomb group proteins; rasiRNA) repeat-associated small interfering RNA; RNAi) RNA interference; siRNA) short interfering RNA; TAS) telomere-associated sequences; TPE) telomeric position effect; UTR) untranslated region.

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of compartmentalization in regulation of gene activity [15, 16]. In meiosis where correct positioning of chromosomes determines correctness of inheritance of genetic material, interaction of chromosomes begins with condensation of telomeres and their certain positioning in the nucleus. There is evidence that satellite like subtelomeric sequences known as TAS (telomere associated sequences) and associated proteins play an important role in these processes [17, 18]. The proteins associated with telomeric and subtelomeric sequences form special telomeric chromatin, and in reality its structure is responsible for all the above-mentioned telomere functions. Interestingly, many components of the telomere–protein complex are highly conservative among organisms employing various mechanisms of telomere elongation. This review considers the structure of the telomeric chromatin in *Drosophila* and highlights the role of its certain components in telomere functioning.

TELOMERE STRUCTURE FORMED BY TELOMERASE

Telomeres formed by telomerase consist of short repeats such as TTAGGG in vertebrates. These repeats form a double stranded DNA sequence of several thousand base pairs and a single strand 3′-end extension (also known as a 3′ single-stranded overhang) of several hundred nucleotides in length. Insertion of the single stand end into the telomeric DNA duplex forms a telomeric t-loop [19]. Figure 1a shows a detailed description of the t-loop. Short telomeric repeats as well as the t-loop are recognized by specific telomeric proteins, representing a platform for formation of more complex telomere–protein complex. This complex stabilizes the structure of the t-loop, protects the chromosome end against degradation, and also controls telomere length by regulating susceptibility of the chromosome end for telomerase. For example, vertebrate conservative DNA-binding telomere proteins TRF1 and TRF2 directly bind the duplex, which consists of telomeric repeats, and interact with proteins stabilizing the t-loop (POT1, RAP1) and also with proteins of the DNA repair system such as heterodimer Ku70/Ku80, MRN-complex (MRE11, RAD50, NBS1), and ATM-kinase [20-22]. Several tens of proteins involved in formation of the telomere complex in mammals and other organisms employing telomerase are recognized. The mode of the complex formation is similar for different organisms, and it includes sequence-specific protein binding with DNA. The resultant complex also acts as a cap protecting the DNA end against the repair system and preventing telomere fusion; it also regulates telomere length. Although regulation of telomere length is a complex process, which depends on numerous factors, it may be simplified to protein titration on telomeric DNA: the decrease in proteins on a shortened telomere

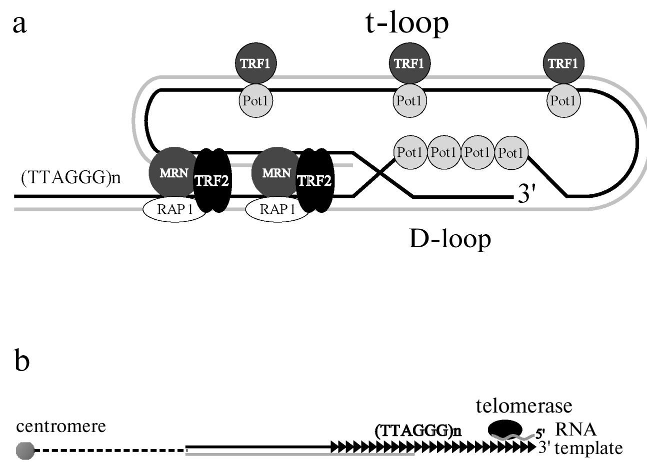

Fig. 1. Schematic presentation of the human telomere. a) Structure of t-loop. A single stranded G-rich tail of human telomere is inserted into the duplex and forms a structure known as the D-loop (D for displacement). Pot1 binds the single-stranded G-rich chain of the D-loop. TRF1 and TRF2 bind the double-stranded sites of telomeric DNA and also attract other proteins of the telomeric complex such as Rap1, MRN complex, and others. b) An "opened" conformation of telomere, at which synthesis of telomeric DNA occurs. Telomerase elongating 3′-end overhang on RNA component template is shown.

increases its susceptibility to telomerase in germinal cells or represents a signal for arrest of somatic cell division [4, 20]. Telomere and RNA template for reverse transcription of telomeric repeats (Fig. 1b) are important factors regulating telomere length. Interestingly, knockout of a gene encoding the RNA component of telomerase (and therefore causing impairment of telomerase functioning) is not lethal, but it causes sterility of mice [23]. These data indicate that telomerase activity and the telomere elongation process are the most crucial in germinal tissues during formation of gametes, the cells involved into formation of a zygote, which then undergoes multiple divisions in the process of formation of a multicellular organism. In *Drosophila*, telomeres are formed due to retrotransposition of specialized telomeric retrotransposons. *Drosophila* telomeric protein complex exhibits surprising similarity with corresponding telomeric protein complex of organisms employing telomerase [24]. In subsequent sections of this review we consider structure–functional principles of organization of telomeric chromatin in *Drosophila*, which are similar to those in organisms expressing telomerase and also specific for *Drosophila*.

STRUCTURE OF *Drosophila* TELOMERES

Drosophila telomeres are formed by transpositions of retrotransposons of three families: *HeT-A*, *TART*, and *TAHRE* (Fig. 2). They all belong to the LINE (long interspersed nucleic elements) type retroelements lacking long

terminal repeats. Transposition of these telomeric elements at the chromosome end is rather specific: although various elements are located randomly, their positioning follows the head-to-tail principle and their poly(A) tails always face proximally. Transpositions of *Drosophila* telomeric elements obviously employ the same mechanisms as in the case of insertion of the LINE-type retrotransposons [25]. Elements of this type are used as primers for DNA synthesis of RNA template, and the 3′-end is formed by endonuclease cleavage of DNA. It is suggested that *Drosophila* telomere retrotransposons can use the 3′-protruding end of the chromosome as a primer, but the mechanism of site-specific transpositions of telomeric retroelements to the chromosome end remains unclear.

In one generation, *Drosophila* telomeres shorten by 70-75 nucleotides [26], whereas transposition of full-size retrotransposon results in chromosome elongation by several thousand bases. Evidently, maintenance of normal length of telomeres requires strict control of transposition frequency. The possibility of transposition of three various elements to the chromosome end and the process of end degradation will result in various terminal nucleotide

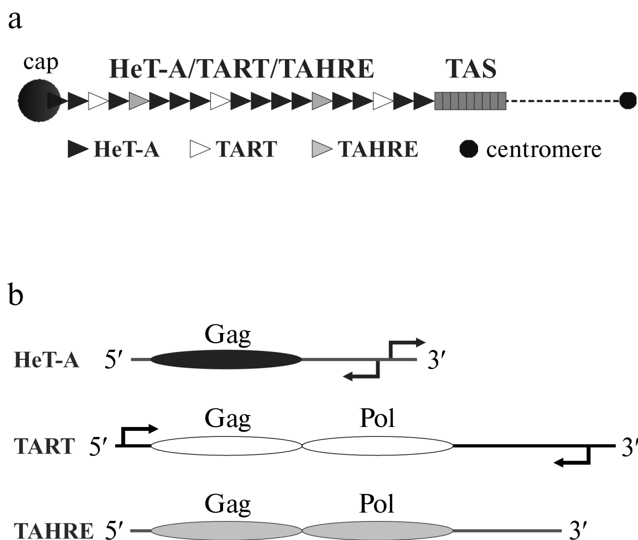

Fig. 2. *Drosophila* telomeres. a) Scheme of the structure of the telomere region. The head-to-tail tandem location of *HeT-A*, *TART*, and *TAHRE* retrotransposons forming telomeric DNA is shown. In the *Drosophila* telomere, the number of *HeT-A* copies predominates. The satellite-like TAS sequences have a relatively proximal location. The protective protein cap-complex is formed at the chromosome end in a sequence-independent manner. b) Scheme of *Drosophila* telomeric retroelements. Arrows indicate promoters and directions of transcription; rectangles correspond to ORF for Gag and Pol proteins. The *HeT-A* promoter located at its 3′-part is involved in transcription of the next copy. *TART* has sense and antisense promoters located at 5′- and 3′-parts, respectively. Promoter of the *TAHRE* element has not been studied yet. *HeT-A* lacks ORF for Pol protein, which contains the reverse transcriptase domain, and therefore this element is a non-autonomic retrotransposon.

sequences both in various individuals and also in various cells of the same individual as well. Such heterogeneity of end sequences distinguishes *Drosophila* telomeres from the telomeres formed by short repeats. It remains unclear whether *Drosophila* telomere ends with the 3′-end overhang, which may form a structure similar to the vertebrate t-loop. The indirect evidence for this possibility is the *Drosophila* telomere elongation due to recombination of tandem repeats located on the same chromosome [27-29]. It is also possible that in this case recombination is facilitated by integration of the chromosome end into the internal region of telomeric DNA. Nevertheless, no evidence for the existence of a stable t-loop in *Drosophila* has been obtained by molecular or cytological methods.

There are different proportions of the retrotransposons *HeT-A*, *TART*, and *TAHRE* in the *Drosophila* telomeres. The main structural component of telomeres is the retrotransposon *HeT-A*; in genomes of *D. melanogaster* strains, there are about 30 copies of this element. The number of *TART* copies is about ten [30, 31]. This is the number of full-length elements capable of encoding a protein. In telomeres there are retroelement copies truncated at the 5′-end. Appearance of such copies may be explained in two ways. Low processivity of reverse transcriptase may cause early arrest of reverse transcription and appearance of an incomplete copy of the retroelement. Truncation of the terminal copy of the retroelement may also occur as a result of end underreplication up to the moment of attachment of the next retroelement. The third telomeric element, *TAHRE*, has recently been found during analysis of the genome sequence of *D. melanogaster* [8, 31]. The genome of a *D. melanogaster* strain sequenced within the genome project contains four copies of *TAHRE*, but only one is a full size copy [8]. Telomeres in the culture of *Drosophila* cells, as well as in cultures of mammalian cells, are shortened and contain 14 copies of *HeT-A* and four copies of *TART*-elements [30].

The retrotransposons *TART* and *TAHRE* (with the size of the longest copies of ~16 and ~11 kb, respectively) contain two open reading frames (ORF), ORF1 and ORF2, and also extended 5′- and 3′-noncoding regions. ORF1 encodes a protein containing “zinc fingers”; this protein is homologous to retroviral RNA-binding Gag protein [32]. ORF2 encodes a protein homologous to Pol protein with endonuclease and reverse transcriptase domains. Polypeptides Gag and Pol of *Drosophila* telomeric retrotransposons demonstrate the highest homology with corresponding proteins of insect LINE type retrotransposons [33]. The *TART* and *TAHRE* ORF2 are highly homologous. The telomeric retrotransposon *HeT-A* (~6 kb) exhibits high homology with *TAHRE*, especially in 3′- and 5′-untranslated regions, but lacks ORF2 encoding reverse transcriptase. Until recently it was believed that the telomeric retroelements *HeT-A* and *TART* are not related to each other [9]. Discovery of a new telomeric element *TAHRE* gave a new look on the evolu-

tion of *Drosophila* telomeric retrotransposons. It is suggested that *TART* and *TAHRE* appeared in the result of divergence of a common ancestor element, whereas *HeT-A* originated from *TAHRE*, but lost the reverse transcriptase gene [8].

Interestingly, the retroelement *HeT-A*, lacking its own reverse transcriptase (in other words a non-autonomic retroelement) is the main structural component of *Drosophila* telomeres. Nevertheless, the vast majority of spontaneous attachments to the chromosome end are determined by *HeT-A* transpositions [27, 34, 35]. It is suggested that the reverse transcriptase *TART* and/or *TAHRE* may be responsible for *HeT-A* transpositions [8, 36]. Indeed, *TART* and *TAHRE* are highly conservative telomeric elements in various *Drosophila* species [37, 38]. This suggests their importance for telomere functioning, possibly as the source of reverse transcriptase for *HeT-A* transpositions. The Gag protein encoded by the element *HeT-A* plays the key role in "delivery" of RNA template of the telomeric retrotransposons to the chromosome end. *HeT-A* Gag is colocalized with the HOAP protein, the component of capping complex in the telomere region of the interphase nucleus [36]. Reasons for specific localization of *HeT-A* element Gag protein remain unclear because the Gag proteins of other transposons (including *TART*) have not been found in the telomere region [36, 39]. However, co-expression of Gag proteins on *HeT-A* and *TART* templates results in colocalization of both proteins in the telomere regions [36]. These results have been used as a background for a hypothesis suggesting cooperation of several retroelements for elongation of the *Drosophila* telomere [9].

The existence of unusually extended 5′- and 3′-untranslated regions (UTR) is a characteristic feature of the telomeric retrotransposons. The *HeT-A* 3′-UTR is more than 3 kb, and in the case of *TART* this parameter is up to 5 kb. Evidently, the elements *TART* and *TAHRE* cannot play a structural role (i.e. to serve as a platform for binding of conservative telomeric proteins) because these elements are not present in all telomeres [7, 8]. Moreover, in various strains of *D. melanogaster TART* and *TAHRE* are located on different chromosome arms ([7], Kalmykova and Savitsky, unpublished results). It is suggested that the sequence of *HeT-A* 3′-UTR containing repeated A-rich sites is involved in binding of proteins, forming a specific telomeric chromatin [9]. However, in contrast to highly conservative vertebrate TRF proteins and their homologs in *Schizosacharomyces pombe* and *Saccharomyces cerevisae* (Taz1 and Rap1, respectively) recognizing telomere repeats formed by telomerase, no protein components specifically binding telomeric retrotransposons have been recognized in *Drosophila*. The size of the *Drosophila* telomere is about 50 kb, and it consists of *HeT-A*, *TART*, and *TAHRE* elements, which are not interrupted by other sequences [30, 31]. How do *Drosophila* telomeres function in the cells, how do proteins bind to them, and what mechanism underlines recognition of heterogeneous telomeric DNA by these proteins?

CAPPING OF *Drosophila* TELOMERES

The components of *Drosophila* capping complex were recognized using genetic methods, which were employed for studies of mutants characterized by telomere fusion effects. These included HP1 (heterochromatic protein 1) [40], HOAP (HP1/ORC associated protein) [41], ATM kinase, the main component of cell response during DNA damage, and also components of the MRN repair complex: MRE11 and Rad50 [42-46] (table). The formation of the telomeric cap at the chromosome end irrespectively to nucleotide sequences of DNA ends is a characteristic feature of the *Drosophila* telomeric cap. As mentioned above, the nucleotide sequence at the end of *Drosophila* telomeres is different in different cells of one individual, and it is also known that terminally deleted chromosomes lacking telomeric and subtelomeric sequences may form a cap of the same compositions as a normal telomere [40]. Such components of the cap as ATM kinase and MRN complex proteins MRE11 and Rad50 are universal constituents in the cap in telomeres formed by telomerase.

HP1 is a highly conservative protein containing the chromo domain, which is involved in formation of inactive chromatin in eukaryotes. The immunolocalization of HP1 on the polytene chromosomes revealed the presence of HP1 binding sites in the chromocenter, along chromosomes, and in the telomeric region [40, 47]. Mutations in the gene *Su(var)2-5* encoding HP1 resulted in frequent telomere fusion and appearance of long linear or circular chromosomes [40]. Interestingly, such effect was caused by HP1 mutations involving the region between the CD (chromo domain) and CSD (chromo shadow domain), so-called H-(hinge) domain; for this domain the DNA-binding activity was demonstrated [47]. It is suggested that HP1 directly links the chromosome ends and initiates cap formation. Mutations in the HP1 chromo domain do not influence its capping capacity [40, 47]. The HOAP protein is the other important cap component; mutations in the gene *caravaggio* (*cav*) encoding HOAP result in the same frequency of the chromosome fusions as mutations of the gene *Su(var)2-5* [41]. The HOAP protein is colocalized within the telomere region with HP1 protein and binds to its CSD- and H-domains [48]. However, in *Su(var)2-5* mutants, some amount of HOAP is localized within telomeres [41]. Both proteins, HP1 and HOAP, bind the ends of terminally deleted chromosomes [24, 40] suggesting sequence-independent mode of binding. The region of telomere cap at polytene chromosomes also binds such heterochromatin proteins as HP2 (a partner of HP1), Su(var)3-7 (a protein of pericentromere chromatin containing zinc fingers), and

Components involved in *Drosophila* telomeric chromatin

Name of protein	Short description of protein and its role in maintenance of *Drosophila* telomeres	References
HP1 (heterochromatic protein 1)	Conservative chromatic protein involved into formation of heterochromatin in various organisms. It contains the domains CD (chromo domain) and CSD (chromo shadow domain) involved in interaction with methylated forms of histone proteins and histone methyl transferases. It is a component of *Drosophila* capping complex	[28, 40, 47]
HOAP (HP1/ORC-associated protein)	DNA-binding protein interacting with HP1. In cytoplasm there is a complex of HP1, HOAP, and ORC (origin recognition complex, which comprises of six subunits, ORC1-ORC6), but ORC2 subunit has not been recognized in the telomere region. It is a component of the capping complex of *Drosophila* telomeres	[41]
MRN	Complex containing components MRE11, RAD50, and NBS1; it is involved in repair of double strand breaks of DNA. MRE11 and RAD50 are shown to be employed in attraction of HP1 and HOAP to telomere	[42, 43]
ATM (ataxia-telangiectasia mutated) kinase	It is involved in cell response to DNA damage; the enzyme phosphorylates the protein involved in repair process. Mutations in the gene encoding ATM cause telomere fusion and decrease in HP1 and HOAP content in the telomere region	[42, 44-46]
Ku70/Ku80	Heterodimer that binds DNA ends; it is involved in repair of double strand DNA breaks. In *Drosophila*, it is a negative regulator of telomere length. Ku70/Ku80 is suggested to bind the end of telomeric DNA	[29]

SUUR (a protein localized in late replicated regions, which is typical for heterochromatin) [49].

Recent studies have demonstrated that mutations in genes encoding components of the repair MRN complex (MRE11 and Rad50) cause impairments in binding of HP1 and HOAP with telomeres of polytene and mitotic chromosomes and also cause chromosome fusions, although to a lesser extent than mutation of genes *Su(var)2-5* and *cav*. Both proteins were uniformly distributed along chromosomes, and their enrichment in the telomere region was not observed [43]. These data presented a good background for the hypothesis that MRN is not a stable cap component, and it binds for short time to the telomeric DNA ends and attracts the proteins of the capping HP1/HOAP complex [24, 43]. The other protein involved in cap formation in *Drosophila* is ATM kinase, the major factor involved in the response to DNA damage. Mutations in the *tefu* (*telomere fusion*) gene, encoding ATM in *Drosophila*, cause telomere fusion; this is accompanied by impairment of HP1/HOAP with the telomeres of the polytene chromosomes [42, 44-46]. The simplest hypothesis is the suggestion that one of the components of the MRN complex is the substrate of ATM kinase; however, this is not right [42], and we do believe that in the near future the unknown components of the biochemical pathway leading to formation of the protective capping complex in *Drosophila* will be recognized.

The heterodimer Ku70/Ku80 is involved in telomere functioning in *Drosophila* as well as in organisms containing telomerase [29, 50, 51]. The deletions of loci encoding the Ku-proteins increased the frequencies in the transpositions of *HeT-A/TART* to the end of terminally deleted chromosome; however, they do not influence expression of the telomere repeats [29]. It is possible that this heterodimer involved in repair of the double stranded breaks of DNA binds the end of telomeric DNA and regulates its susceptibility for transpositions of the telomeric DNA in *Drosophila* [29].

In spite of the progress achieved in studies of formation of the capping complex in *Drosophila*, this field still has more problems than clear answers. Evidently, mechanisms responsible for assembly of the capping complex differ in different cells, particularly in terminally differentiated somatic cells (the polytene chromosomes of salivary glands) and mitotic and meiotic cells. For example, mutation in the gene *tefu* encoding ATM did not influence localization of HOAP in the telomeres of mitotic chromosomes, but decreased the number of HOAP and HP1 in the telomeres of salivary gland polytene chromosomes [42, 44]. The meiotic chromosomes were not analyzed in the studies devoted to investigation of mechanisms underlying *Drosophila* telomere capping. The functional load onto the telomeres is different in dividing precursors of germinal cells and in terminally differential cells; in the former case, telomere elongation occurs and this obviously is accompanied by facilitated access to the chromosome end for terminal transpositions, in the latter case, inactive condensed chromatin assembles to protect

chromosome end against degradation. Similarly, telomerase activity and the structure of the telomere chromatin are strictly regulated depending on the stage (G1, S, G2) and type (mitosis or meiosis) of cell cycle and also in response to DNA damage and telomere shortening [52]. In mammals, telomerase is stimulated in actively proliferated germinal tissues. Detection of structural differences in the telomeric chromatin, particularly in the structure of the capping complex at various stages of development, will clarify functional time course of telomeres in various organisms irrespectively of the mode of telomere elongation.

CHROMATIN ORGANIZATION OF SUBTELOMERIC SEQUENCES

The telomere-associated sequence (TAS) is a region that is proximally localized versus telomeric repeats; it consists of satellite-like repeats, fragments of mobile elements. TAS differs in length and sequence not only among different species but also among chromosomes within one species. In most organisms (including man), TAS is characterized by heterochromatin structure [53]. The function of this rather extended chromosome region remains unclear. Cytologically in *Drosophila* salivary gland polytene chromosomes the subtelomeric region looks like a more condensed site compared with a decondensed structural region resembling euchromatin and containing telomeric retrotransposons [54]. In *Drosophila* satellite-like sequences of 0.4-1.8 kb form TAS arrays of 15-25 kb on various chromosome arms [17, 31, 55-57], and TAS of left arms of the second and the third chromosomes (2L/3L) and TAS of right arms of the second and the third chromosomes and also X chromosome (2R/3R/X) share similarity. The small fourth heterochromatin chromosome of *Drosophila* has special TAS structure; these are fragments of various mobile elements [17].

One of the most widely used methods for determination of chromatin structure in a particular locus employs expression of a reporter gene during transgene insertion into the genome locus under study. Inactivation of the reporter gene during transgene insertion to the subtelomeric region is referred to as telomeric position effect (TPE). In the case of *Drosophila*, it has been demonstrated that the reported gene *white* determining eye color becomes silenced after insertion into TAS. In contrast to the classic position effect related to suppression of a gene integrated into heterochromatin, *Drosophila* TPE does not depend on HP1 and known modifiers of the position effect excluding the fourth chromosome, where TPE depends on HP1 [17]. This is not surprising because the preferentially heterochromatinized fourth chromosome is cytologically located in the chromocenter, the region enriched with heterochromatin proteins. The TAS sequence of other chromosomes contains motifs recognized by some Polycomb (PcG) proteins [17, 49, 58]. These DNA-binding proteins are involved in regulation of activity of genes determining development in eukaryotes. Some of them are repressors, others are activators of gene transcription, and they act at the level of changes in chromatin structure [59]. In contrast to HP1 localized at the very end of a chromosome, immunostaining of the polytene chromosomes recognized PcG protein binding to the subtelomeric region [49, 58]. Double staining for HP1 and PcG proteins clearly demonstrated non-overlapping pattern in their localization: HP1 was associated with a cap structure at the chromosome end, whereas PcG proteins were associated with TAS [58]. The region containing *HeT-A/TART/TAHRE* remained unstained; this suggests that these proteins are not involved in chromatin formation in the region of telomeric repeats. Thus, chromatin structure in the subtelomeric region is determined by the type of TAS. Interestingly, translocations of the second chromosome TAS to the fourth chromosome and *vice versa* retained specific structure of chromatin and TPE features in the translocated regions typical for their initial localization [17]. Being positioned on the second chromosome within a translocated fragment, transgenes inserted in the fourth chromosome TAS retain dependence of TPE on HP1 within the translocated fragment, and translocation of the terminal region of the second chromosome to the fourth did not influence the dependence of expression of transgenes in TAS region on PcG proteins. However, in the case of translocation of the fourth chromosome to the second chromosome there was a sharp decrease in TPE-induced transgene silencing [17]. This phenomenon may be attributed to changes in nuclear localization of the translocated fragment: usually the fourth chromosome is positioned in the chromocenter enriched with heterochromatin proteins, whereas translocation transfers the nucleotide sequence to the periphery of the nucleus. These results suggest that TAS provides sequence-specific assembly of protein complexes involved in larger protein compartments of the nucleus. Based on these data, it is tempting to propose that TAS play the key role in processes related to telomere positioning inside both the interphase and mitotic nucleus.

In many organisms it has been demonstrated that chromosome arrangement within the interphase nucleus chromosomes involves centromere and telomere positioning at opposite poles of the nuclear periphery (so-called Rabl configuration originally described by C. Rable [60]). Although there is increasing evidence for existence of chromosome territories, it still remains unclear whether chromosomes always occupy strictly determined space in the interphase nucleus of particular cells [15]. Intranuclear localization of chromosomes has been well studied in mitotic and meiotic cells due to good visualization of condensed chromosomes under the microscope. Clustering of telomeres in meiosis is a universal conserva-

tive stage of meiotic prophase often referred as the bouquet stage because chromosomes are gathered together and the chromosome loops resemble a bouquet. This is a key stage in initiation of the homolog pairing process followed by subsequent chromosome segregation [61]. Impairments at this stage result in incorrect chromosome segregation. In some organisms (e.g. in yeasts) telomere clustering occurs in mitosis. In yeasts mutations of some proteins of the telomeric complex result in impairments of nuclear localizations of chromosomes. For example, a heterodimer of Ku proteins determines binding of yeast telomeres with nuclear membrane [15, 62], whereas *S. pombe* Taz1 recognizing the telomeric repeats and involved in control of telomere length interacts with the same subtelomeric repeats; this provides a link between telomere and centrosome proteins during meiosis even in the absence of telomeric repeats [18]. Thus, the chromatin telomeric complex is a multifunctional structure, and the same protein component of this complex may be involved into various processes. However, the complete "picture" of functioning of this complex remains unclear.

MODIFICATION OF HISTONES IN THE TELOMERIC REGION OF *Drosophila*

Study of modifications of histones, which are markers of chromatin status, attracts special interest in terms of analysis of chromatin structure [63]. Histone modifications typical for both open and condensed forms of chromatin have been recognized in telomeric regions of *Drosophila*. Histone H3 methylated at lysines 9 and 27 (H3MeK9 and H3MeK27, respectively) is a marker of heterochromatin. H3K9 methylation involves activity of methyltransferase Su(var)3-9, whereas methylation at H3K27 involves E(z) [64, 65]. The chromo domain of HP1 binds H3MeK9 and interaction of HP1 CSD domain with methyl transferases causes subsequent methylation of histones, and these interactions resemble a chain reaction, which is believed to be a basis of heterochromatinization [66]. H3Me3K4 is a histone modification typical for active chromatin. Di- and trimethylated forms of H3K9 (H3Me2K9 and H3Me3K9, respectively), H3MeK27, and H3Me3K4 have been found in the telomeric region [47, 49, 65].

The *Drosophila* telomere can be subdivided into three regions by histone modifications: a cap, telomeric retrotransposons, and TAS. The clearest situation is in the subtelomeric region containing TAS. In the region of subtelomeric repeats, chromatin is enriched with H3Me3K27 due to activity of histone methylase E(z) [49, 65]. E(z) is a member of the Polycomb group proteins; it exhibits chromomethylase activity with respect to lysines at the 9th and 27th positions of histone H3 [65]; methylation at these positions is a label that attracts repressor protein complexes and inactivation of genes involved in development [67]. In the subtelomeric region, H3Me3K27 is a substrate for binding of Polycomb group proteins [49, 58].

The presence of H3Me3K9 in the distal region of a telomere, including the cap and telomeric retrotransposons, is determined by activity of some unknown methyl transferase: mutations of genes encoding chromomethylases Su(var)3-9 and E(z) did not influence H3K9 methylation in these regions [47]. On the other hand, mutation in the chromo domain of *Su(var)2-5*, which did not influence HP1 binding at the chromosome end, was accompanied by disappearance of H3Me3K9 from telomeres [40, 47]. Based on these data, is has been suggested [47] that HP1 binds the end of chromosome DNA via its H-domain, forms a cap, and then interacts with some unknown chromomethylase via its CD and CSD. This causes spreading of HP1 over telomeric DNA. However, there are experimental data that are inconsistent with this hypothesis of spreading of HP1 containing heterochromatin protein complex over the telomere. Fine cytologic staining demonstrates HP1 localization at the very end of the chromosome, which obviously does not involve the region of *HeT-A/TART/TAHRE*-arrays [49, 58]. Immunostaining of H3Me3K9 revealed discrete bands in the region of telomeric retrotransposons on polytene chromosomes lacking HP1 [49]. Molecular studies provide more accurate limits of HP1 distribution on the chromosome end; these are evaluated by a distance of less than 1 kb (Savitsky and Kondrat'ev, personal communication). Interestingly, the form of histone H3Me3K4 typical for actively transcribed genes has also been found in the region of telomeric repeats [49]. Below we will discuss data on decondensed chromatin in the region of telomeric repeats. In mammals protein homologs of HP1 are also components of the telomere complex. Although their binding with telomere depends on activity of methylase Suv3-9h, mutation in a gene encoding this enzyme does not result in the effect of chromosome fusion [68]. It is possible that HP1 homologs play a structural role in formation of the heterochromatin telomere in mammals and they do not represent obligate components of the capping complex.

STRUCTURE OF *Drosophila* CHROMATIN IN THE REGION OF *HeT-A/TART/TAHRE* REPEATS

The sequence of telomeric repeats synthesized by telomerase is a platform for binding of specialized DNA-binding telomeric proteins, which represent a basis for subsequent assembly of the whole telomere complex. However, no proteins specifically bound sequences of telomeric retrotransposons forming telomeric DNA of about 50 kb per telomere have been found so far in *Drosophila* telomeres [30, 31]. It is suggested that the A-

rich sequence of extended 3′-UTR of *HeT-A* element may be recognized by specialized proteins [9]. Even if this is true, chromatin in the region of telomeric retrotransposons is not condensed (in contrast to the region of telomeric repeats formed by telomerase). There are several examples of transgene integration into coding and promoter regions of *HeT-A*, *TART*, and *TAHRE*. In most cases, when the transgene has been inserted into an internal region of the telomeric repeats, normal activity of a reporter gene is observed irrespectively to particular positioning of the insert: coding or UTR region of each of three elements [54]. Only when the transgene is integrated into an element flanking TAS reported gene silencing is observed [54]. This effect has also been shown earlier, and it is obviously attributed to spreading of proteins localized in the heterochromatin region of TAS to the flanking regions and this is accompanied by silencing of genes located there [35, 69]. The longer the *HeT-A/TART/TAHRE* array on the chromosome end, the higher the expression of the reporter gene located between TAS and telomeric retroelements is [35]. It has thus been concluded that chromatin of the *HeT-A/TART/TAHRE* arrays exhibits an activation effect on flanking regions. Cytologic studies characterize the region of telomeric retroelements as a zone of decondensed chromatin similar to euchromatin [49, 54]. Proteins typical for decondensed form of chromatin (e.g. phosphokinase JIL-1, DNA-binding protein Z4, and H3Me3K4) are localized in the region of telomeric retrotransposons [49].

Thus, three chromatin domains are recognized in the *Drosophila* telomere: the first is involved in formation of the protein cap, the second is the heterochromatin domain in the TAS region, and the third domain is decondensed chromatin in the region of *HeT-A/TART/TAHRE* arrays [49, 54]. This distinguishes *Drosophila* telomeres from heterochomatic nucleoprotein complexes in organisms employing telomerase. A characteristic feature of *Drosophila* telomeres consists in the fact that the RNA-template for telomere elongation is copied from the telomeric sequences (in contrast to telomerase RNA-component encoded by a separate cell gene). Consequently, the problem of chromatin structure in the region of *Drosophila* telomeric repeats basically represents the problem of transcriptional regulation of *HeT-A*, *TART*, and *TAHRE* retrotransposons.

Promoter of *HeT-A* located in the 3′-region is involved in expression of a flanking element [70] (Fig. 2b); this is a characteristic feature of *HeT-A* transcription. The last 300 bp of 3′-UTR-*HeT-A* exhibits promoter activity [27]. In a transgenic construct, *HeT-A* promoter is active in euchromatin sites, but increase in the promoter region up to 600 bp results in reporter gene silencing; this suggests a possibility of binding of protein repressors in this region [71]. Protein product of gene *prod* (proliferation disrupter) might be one of these proteins; mutations in this gene are accompanied by increased expression of *HeT-A* [72]. *HeT-A* is actively transcribed in germinal tissues and actively replicating cells, for example, in larval imaginal discs that will make up adult tissues [71].

The existence of sense and antisense promoters (with the antisense activity exceeding that of the sense one) is characteristic for *TART* transcription [73]. The start sites of sense and antisense *TART* transcription are located in 5′- and 3′-UTR in the region of extended nonterminal repeats typical for this element [74]. Functional importance of symmetric *TART* transcription in the living cycle of the retrotransposon remains unclear. Involvement of antisense RNA in the retroelement replication is suggested in the case of the retrotransposons *Dre* (in *Dictyostelium*) and *TOC1* (*Chlamydomonas*), which share similar structure [75, 76]. The homology of 3′-UTR elements of *HeT-A* and *TAHRE* suggests the existence of a *TAHRE* promoter in its 3′-region; however, experimental evidence supporting this suggestion still remains to be obtained.

There is little information about transcription factors influencing activity of the telomere retroelements. Although there are some signs typical for open chromatin, the active form of RNA polymerase (PolIIo) is absent in the region of telomeric repeats in polytene chromosomes [49]. This suggests that transcription of retrotransposons was repressed. Indeed mutation of several genes encoding components of telomere protein complex abolished silencing and caused derepression of *HeT-A* and *TART* expression. Mutations in *Su(var)2-5* gene encoding HP1 caused significant accumulation of *HeT-A* and *TART* transcripts; this was accompanied by increased frequency of telomeric retrotransposon additions to the chromosome end [28]. Thus, HP1 is a negative regulator of the telomere length in *Drosophila*. Taking into consideration its role in cap formation, one may suggest that HP1 determines availability of the chromosome end for retrotransposon addition. The method of immunolocalization does not recognize HP1 in the region of telomeric repeats [40, 49, 58]. Thus, the role of HP1 protein in the mechanism of regulation of *HeT-A* and *TART* expression remains unclear. Some increase in the *HeT-A* expression level was observed in double mutants by genes encoding ATM and ATR kinases, the main participants of cell response to DNA damage and components of *Drosophila* capping complex [77]. The Ku proteins, which are negative regulators of the telomere length, are not involved in regulation of expression of telomeric retrotransposons [29]. There are results indicating lack of influence of genes involved in RNAi on *HeT-A/TART* expression in *Drosophila* larvae [47]. It is important to emphasize that in all these cases expression of the telomeric repeats was studied using RNA preparations isolated from adult flies or larvae consisting preferentially of somatic tissues. We have discussed above that telomeres play different roles during various stages of the cell cycle. Our data suggest that control of expression of

telomeric retrotransposons and telomere length occurs in germinal cell precursors where this process is especially important, and it employs a mechanism closely related to RNAi [78]. In the next section we will consider data on involvement of RNAi in silencing of the telomeric transposons and discuss an important role of RNAi components in formation of telomere protein complex during meiosis.

ROLE OF RNAi IN REGULATION OF EXPRESSION OF TELOMERIC RETROTRANSPOSONS AND FREQUENCY OF THEIR TRANSPOSITIONS TO THE CHROMOSOME END IN GERMINAL TISSUES

RNA interference (RNAi) is a mechanism of post-transcriptional gene silencing induced by appearance of homologous double-stranded RNA (dsRNA) in the cell. In *Drosophila*, an RNaseIII-like enzyme called Dicer processes long dsRNAs into small, short interfering RNA (siRNA). One of strands of these siRNAs functions in the RNA induced silencing complex (RISC). Proteins of the Argonaute protein family, which interact with short RNAs and an RNA target, are the main components of RISC. RISC binds the mRNA target and cleaves it in the region complementary to siRNA. The miRNAs (21-22 nucleotides) formed during processing of hairpin RNAs (encoding by cell genes) cause translational inhibition of complementary mRNAs [79]. The miRNAs play an important role in the regulation of gene activity during development [80]. The system of RNAi causes silencing of viral gene expression and mobile element expression in various organisms. *Drosophila* telomeric retrotransposons are one of a few examples of acquisition of vitally important functions in the genome by parasitic elements. However, telomeric retrotransposons also represent a target for the RNAi system. It is not a surprising phenomenon because during one generation *Drosophila* chromosomes shorten by 70-75 bp, and one addition provides elongation by 5-15 kb. The RNAi system suppresses excessive retrotransposon activity and maintains transcripts at low level because this mechanism implies the presence of sense and antisense transcripts acting as triggers of this system. Mobile elements, the potential source of dsRNA, are the target of the mechanism of RNAi in various organisms, but pathways of processing of their dsRNAs and a putative effector pathway still require detailed investigation. Only some components of the RNAi system involved in silencing of retrotransposons have been identified. Detection of short retrotransposon specific RNAs called repeat-associated small interfering RNA (rasiRNA) suggests a putative role of a mechanism similar to RNAi in the regulation of their expression. In contrast to siRNA and miRNA (of 21-23 nucleotides in length), rasiRNAs are a bit longer (25-29 nucleotides). Mutations in genes encoding proteins involved in metabolism of siRNA (Dcr2, Ago2, R2d2) and miRNA (Dcr1, Ago1, Loqs) did not influence processing/stability of rasiRNA [81]. Specific features of RNA silencing of retroelements and cell repeats suggest existence of a third biochemical pathway (after siRNA and miRNA) related to silencing of repeated genes including retrotransposons [81]. The main characteristic feature of this pathway is its functioning in germinal tissues. The transcripts *HeT-A*, *TART*, and *TAHRE* are accumulated in germinal tissues of females containing mutations of genes *spn-E* (encoding RNA helicase) and *aub* and *piwi* (encoding proteins of the Argonaute family) ([78, 81, 82], Shpiz and Kalmykova, unpublished data). All these components are involved in silencing of retrotransposons and repeated genes in germinal tissues [81-85]. Short RNAs specific for *HeT-A*, *TART*, and *TAHRE* are 25-29 nucleotides long. Mutations of genes involved in siRNA and miRNA pathways did not influence either quantity of short RNAs or the level of expression of telomeric elements ([81], Kalmykova, unpublished results). Thus, expression of the telomeric retrotransposons *HeT-A*, *TART*, and *TAHRE* is obviously regulated via the rasiRNA pathway.

Frequency of attachments of *HeT-A* and *TART* in RNAi mutants was investigated using a genetic system that phenotypically monitored transpositions of telomeric elements to the end of terminally deleted X-chromosome [27, 78]. In the strain carrying mutant alleles of *spn-E* or *aub* in heterozygotes (homozygous mutants are sterile), the frequency of additions was 100 times higher than in a strain without mutations. Interestingly, a vast majority of telomere additions was determined by transpositions of *TART* element; mutations of the gene encoding HP1 and deletions of genes encoding Ku70 and Ku80 investigated in this system earlier caused increased frequency of additions of *HeT-A* element [28, 29]. It is possible that *TART* is a primary target of RNAi, because its activation requires just one dose of the mutant gene. High frequency of *HeT-A* attachments to the chromosome end was detected only in ovaries of sterile females containing RNAi gene mutations in homozygous state [78]. *TART* was shown to form both sense and antisense transcripts, and the latter predominated [73]. This suggests that *TART* may be a source of a significant amount of dsRNA.

The sense *HeT-A* promoter is located in the 3′-end of the element, and the 3′ sequence of one element directs transcription of its downstream neighbor [70]. Start of antisense transcription is also located in the 3′-end of *HeT-A* (Kalmykova, unpublished data). Although the level of *HeT-A* antisense transcription was rather low and such transcripts were not detected by Northern and *in situ* RNA analyses [73, 78], antisense short *HeT-A* RNAs were detected by Northern analysis using a single strand probe [78]. Disappearance of short RNAs specific for

HeT-A and *TART* correlated with increased frequencies of their transpositions to the chromosome ends in mutants *spn-E* suggests that a mechanism similar to RNAi is also involved in control of telomere length in *Drosophila* germinal cells. Mutations of RNAi genes did not influence localization of the telomere protein components and the level of expression of *HeT-A* and *TART* in somatic cells [47, 78].

However, the mechanism of RNAi-dependent silencing of telomeric retroelements remains unknown. The biochemical pathway linked to siRNA may cause both posttranscriptional degradation of mRNA and also formation of inactive chromatin in a homologous locus, i.e. transcriptional silencing [86]. In plants there is RNA-polymerase IV, which exhibits affinity to heterochromatin sites and can transcribe them. This enzyme is associated with RNA-dependent RNA polymerase (RdRp). Such transcription results in formation of dsRNA, which in turn serves as a substrate for formation of short RNAs. In a protein complex containing an Argonaute family protein as the major component, short RNAs attract histone methyl transferases to homologous sequences and form an epigenetic tag typical for heterochromatin [87, 88]. In plants transcription silencing involves RNAs (24-26 nucleotide-long) that are formed in the nucleus due to activity of one of the Dicer proteins called DCL3 [89]. In plants it has been demonstrated that such RNAs specific for mobile elements may also be involved in DNA methylation [90].

The RNAi system is known to be involved in formation of centromere heterochromatin in yeast *S. pombe* [91, 92]. The yeast centromere consists of the central region flanked by inverted repeats, which consist of tandem copies of repeats related to mobile elements. Forward and reversed transcripts and also short RNAs corresponding to these repeats have been found. Transgenes integrated into the region of centromeric repeats are repressed. Mutations in genes encoding such RNAi proteins as DICER, ARGONAUTE, and RdRp cause the decrease in H3MeK9 (histone H3 modification typical for heterochromatin) and Swi6 (HP1 homolog) in the centromere region. This results in activation of transgenes located in this region [92]. RNAi proteins are necessary for normal functioning of the centromere. Lack of these proteins causes impairments of chromosome segregation in mitosis and related defects in assembly of cohesin complex on the centromere [93].

The mechanism of rasiRNA-dependent silencing of *Drosophila* mobile elements and repeated genes remains unknown. Subsequent studies will answer the question at which level (transcriptional or posttranscriptional) regulation of mobile element expression occurs. This problem is especially interesting for telomeric retrotransposons, because its solution would give a clear answer whether RNA dependent silencing contributes to formation of telomeric chromatin.

RNAi AND TELOMERE DYNAMICS IN MEIOSIS

RNAi-dependent regulation of telomeric retroelement expression occurs in *Drosophila* ovarian germinal cells at the stage of meiotic prophase. This is the longest stage of the first meiotic division, which includes conjugation of homologous chromosomes following by their subsequent condensation and segregation. These processes provide correct transmission of genetic material to offspring. In many organisms, conjugation of homologs begins in the telomeric regions of chromosomes, attached to the nuclear envelope at the stage of the chromosome bouquet; it is characterized by formation of special synaptonemal complex, which glues the two homologs together along their lengths [94]. In organisms employing telomerase, proteins interacting with the telomeric repeats are involved in the process of telomere clustering. For example, it has been demonstrated that the presence of the telomere binding protein Taz1 (in *S. pombe*) and TRF2 (in vertebrates) determines telomere attachment to the nuclear membrane in meiosis. In yeasts, meiotic clustering depends on activity of histone methyl transferase Clr4 [95]. Formation of functional telomeric chromatin is evidently essential for telomere clustering in meiosis.

Cytologic studies have shown that in *Drosophila spn-E* and *aub* RNAi gene mutants there is nondisjunction of autosomes and sex chromosomes in meiosis resulting in decreased fertility [96]. In organisms employing telomerase, impairments in the RNAi system also cause defects in meiotic behavior of telomeres. In *S. pombe*, mutations of RNAi genes caused impairments in telomere clustering in meiosis [93, 97]. Although Taz1 and Swi6 proteins were found in meiotic telomeres of the mutants, formation of the bouquet (i.e. telomere clustering in one region of the nuclear periphery) did not occur; cytologically, telomeres of various chromosomes were detected as discrete structures [93, 98]. In yeast telomeres sequences recognized by the RNAi system are unknown. Nevertheless, it has been demonstrated that the RNA Induced Transcriptional Silencing complex (RITS) [99] and Rdp1 (RNA-dependent RNA polymerase) are localized on the yeast telomere [97]. In *Tetrahymena* mutation of *Dcl1p* gene encoding one of three orthologs of RNase III enzyme Dicer, specific for germinal cells, caused serious impairments in meiotic chromosome behavior [100]. The chromosome bouquet formation preceding homologous pairing did not occur in meiotic prophase, and this resulted in impairments of chromosome segregation and higher lethality of offspring.

Evidently, the RNAi mechanism is involved in formation of meiotic telomere chromatin in various organisms. Obviously, short RNAs in complexes containing Argonaute family proteins participate in attraction of chromatin proteins to the homologous locus (telomeric sequences); these chromatin proteins may represent a

platform for assembly of the synaptonemal complex. Data on involvement of RNAi components in meiotic telomere clustering in *S. pombe* and *Tetrahymena* suggest a universal role of RNAi in telomere functioning in various organisms irrespective of the mode of telomere elongation. Telomere and/or subtelomeric arrays of all organisms contain repeating sequences, which are the potential source of dsRNA and the target for RNAi. *Drosophila* TAS is also the target for RNAi in female germinal cells (Konstantinov, Kalmykova, and Gvozdev, unpublished results). Such a mechanism may operate in other sites of chromosome pairing in meiosis; these sites are known to be enriched with repeating elements (e.g., *Drosophila* rRNA locus) [101]. Although participation of RNAi in formation of telomere chromatin has to be proved, there is evidence that the mechanism of RNA silencing plays a very important role for telomere functioning in gamete precursor cells in various organisms.

Although the nucleoprotein telomere complex has certain functional differences in various organisms, it is a well-adapted system for maintenance of telomere homeostasis. Evidently, cell types and stages of the cell cycle determine the composition of this complex; they definitely have some functional “requests” to the telomere. Studies of *Drosophila* telomeres have shown that in spite of “unusual” mode of telomere elongation, many components of the telomeric chromatin are conservative in various organisms. Based on limited data on the involvement of RNAi in telomere functioning, it is relevant to assume that the role of RNAi in *Drosophila* is not limited to silencing of telomeric retrotransposon expression. In *Drosophila*, regulation of telomere repeat expression involves components of special RNAi pathway, related to rasiRNA and specific for germinal tissues. In somatic cells, RNAi did not influence expression of telomere retrotransposons. Data on involvement of RNAi components in meiotic dynamics of telomeres in *S. pombe* and *Tetrahymena* indicate a special role of RNAi mechanisms in telomere functioning in various organisms. We suggest that any telomeric repeats can be a target for RNAi and a messenger in assembly of protein complexes. RNAi is evidently involved in telomere functioning in gamete precursor cells at the stage of meiosis: it determines chromosome positioning in the nucleus, homolog pairing, and in *Drosophila* it also controls telomere length. Study of functional relationship of RNAi and the components of telomere complex in germinal cells represents a productive approach for investigation of meiotic behavior of telomeres.

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