Chapter 7

From Micronucleus to Macronucleus: Programmed DNA Rearrangement Processes in Ciliates Are Regulated Epigenetically by Small and Long Noncoding RNA Molecules

F. Jönsson

University of Witten/Herdecke, Witten, Germany

Chapter Outline

1. INTRODUCTION

Ciliates, unicellular eukaryotes, have developed into powerful model systems for studying pathways involved in genome remodeling processes. Very uniquely, ciliates have evolved a separation of germline and soma by differentiating two morphologically and functionally different types of nuclei, the micronucleus (mic) and the macronucleus (mac), in the same cell [1]. In the course of developing a new mac, extensive DNA rearrangement processes have to take place including DNA amplification, DNA fragmentation and elimination, in some species reordering of sequences, and, finally, de novo telomere addition. More details on the regulation of these processes have been elucidated over the years showing a strong connection to processes regulated by noncoding RNA (ncRNA). Variation in nuclear organization between different ciliate classes, such as the Oligohymenophoreans (eg, *Tetrahymena*) and the only distantly related Spirotrichs (eg, *Oxytricha* and *Stylonychia*) is reflected in different adaptations of these regulation processes and shows the huge power of ncRNA-regulated mechanisms in genome dynamics.

In ciliates, diploid, generative mics are required for sexual reproduction and therefore are often considered as analogs of "germline" nuclei. During vegetative growth (asexual reproduction by binary fission), mics are transcriptionally almost inactive with their DNA being organized in heterochromatic structures (Fig. 7.1A and B).

The macs, on the other hand, are DNA rich and transcriptionally highly active during vegetative growth, supplying the cell with all transcripts required for its maintenance and vegetative growth [1]. The macs therefore often are referred to as somatic nuclei. In Fig. 7.1A, a vegetative *Stylonychia* cell was stained with antibodies against acetylated histone H3. These histone H3 acetylations are connected with permissive chromatin, showing the transcriptionally active state of macs in the

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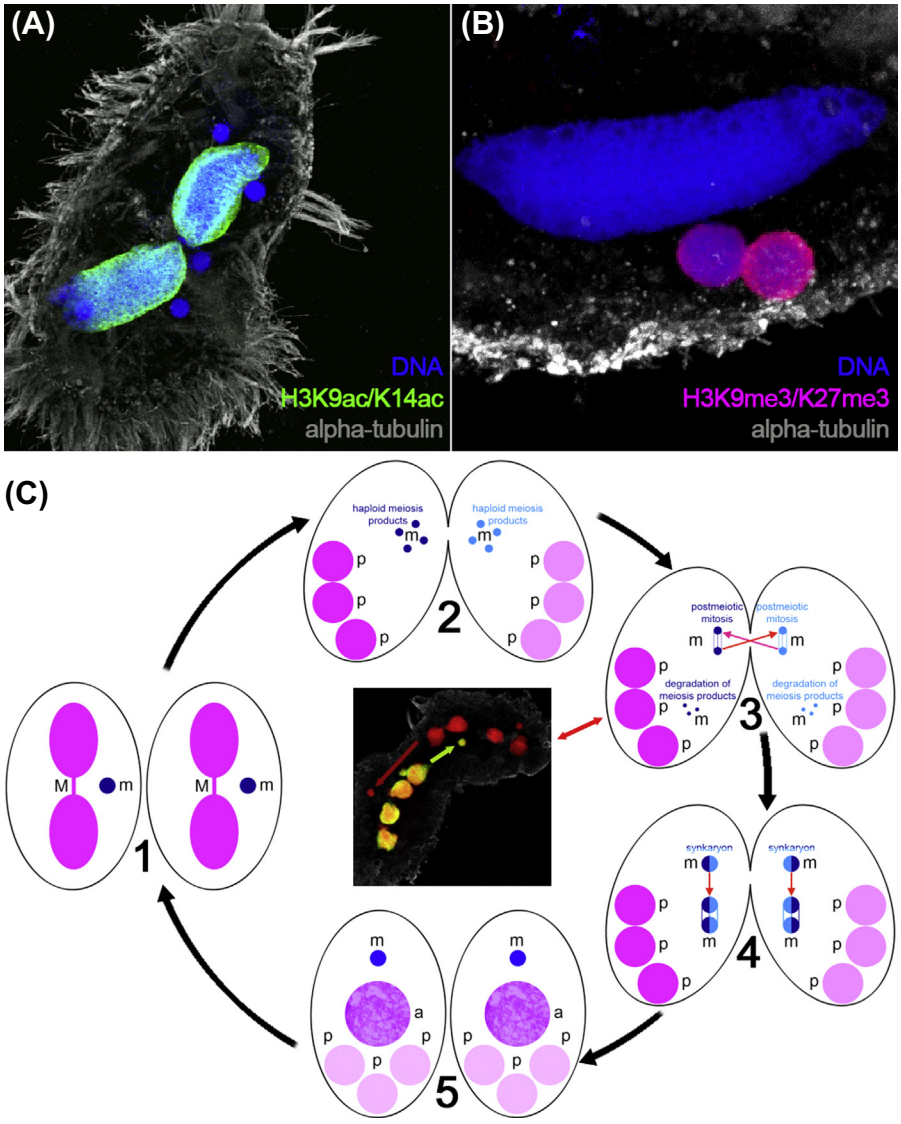

FIGURE 7.1 Nuclear dimorphism in the stichotrichous ciliate *Stylonychia lemnae* and its sexual life cycle. (A,B) Nuclear dimorphism in vegetative *Stylonychia* cells, in both panels DNA was counterstained in *blue* showing several micronuclei as well as the macronucleus which consists of two parts connected by a thin nucleoplasmic bridge (A), cellular shapes are visualized in *gray* by using an α-tubulin antibody. In (A) permissive chromatin of the macronucleus (*green*) was detected by staining with H3K9ac/K14ac antibodies. In (B) transcriptionally inert, heterochromatic micronuclei are stained with H3K9me3/K27me3 (*pink*). (C1–5) Schematic diagram of conjugation in ciliates *(Modified after Grell KG. Protozoology. Berlin, Heidelberg, New York: Springer Verlag; 1973.)*. For explanation see the text. The microscopic panel shows two conjugating *Stylonychia* cells. DNA was stained in *red*. In one conjugation partner nuclei were furthermore marked by incorporation of bromodeoxyuridine in *green*.

vegetative cell [2]. The macs differentiate from derivatives of mics in an elaborated developmental process, resulting in the removal of specifically selected sequences from the developing mac while all sequences encoding genes and regulatory sequences required for their transcription and replication are retained. This elimination of specific sequences can be seen as most extreme form of gene silencing and its regulation shares features with processes involved in RNAi silencing [3,4].

The number of mics and macs in one cell varies between different ciliate species and during the stages in their life cycle. While mics divide by conventional mitosis during vegetative growth, macs undergo a process called amitosis during which they divide without spindle formation or apparent chromosome condensation [5] resulting in daughter nuclei which obtain roughly but not necessarily the same amount of DNA.

2. THE SEXUAL LIFE CIRCLE OF CILIATES

During sexual reproduction (conjugation), which is induced by mixing ciliates of two different mating types, a new mac is generated from a micronuclear derivative. Fig. 7.1C shows the events taking place during conjugation of stichotrichous

ciliates as, for example, *Oxytricha* or *Stylonychia*: first, the mic (m) of each conjugating cell undergoes a meiotic, followed by a mitotic division resulting in four haploid mics (Fig. 7.1C2). Then, in each conjugation partner one of these haploid mics divides mitotically into a stationary and a migratory nucleus, while the other three haploid mics disintegrate. Via a cytoplasmic bridge, connecting the two conjugation partners during conjugation, both migratory nuclei move into the other partner (Fig. 7.1C3) and fuse with the stationary nucleus forming a diploid zygotic nucleus (synkaryon) (Fig. 7.1C4). After mitotic division of the zygote nucleus, one of the daughter nuclei becomes the new mic, while the other differentiates into a new mac (M). During this differentiation process the developing mac is also called macronuclear anlage (a) (Fig. 7.1C5).

In order to differentiate a new mac from the zygotic nucleus the genome has to undergo extreme DNA processing processes, including DNA fragmentation, DNA elimination, DNA reordering (in some ciliate species), and DNA amplification processes [1]. As a first step of macronuclear differentiation the genome of the macronuclear anlage is endoreplicated to a copy number specific for each ciliate species. These endoreplicated chromosomes then become fragmented and a large part of sequences becomes eliminated. This sequence elimination is a very specific process ensuring that all sequences encoding genes and their regulatory sequences remain in the new mac. Depending on the ciliate species, the amount of sequences eliminated during macronuclear development varies. Remaining sequences in the developing macs are thereby processed into short DNA fragments, with the average size and copy numbers of these fragments varying between different ciliate species. To each of these newly formed macronuclear fragments, telomeric sequences are added de novo and (at least in stichotrichous ciliates) in a second amplification process, these macronuclear fragments are amplified, each to its specific copy number [6,7]. Early in conjugation, the old mac (parental mac (p)) is first fragmented and later starts to disintegrate until it disappears from the cell (Fig. 7.1C). Therefore, during a large part of conjugation, the parental macs as well as the anlage coexist in the same cell. This, as discussed later, enables the conjugating cell to exchange factors between these two nuclei and thereby epigenetically transmit information from parent to its offspring [8].

In the following, similarities and differences in the genome structures of the two different ciliate classes, Oligohymenophorea (eg, *Tetrahymena*) and Stichotrichia (eg, *Oxytricha* and *Stylonychia*) are described and discussed in the context of different models proposed for the epigenetic regulation of genome rearrangements in these ciliates.

3. ORGANIZATION OF THE MICRO- AND MACRONUCLEAR GENOMES

In mics, as in conventional eukaryotic nuclei, the genome is organized in long chromosomes with the genes being scattered along the DNA molecules and separated by long stretches of intergenic DNA. A 2014 draft assembly of micronuclear sequences of the stichotrichous ciliate *Oxytricha trifallax* estimated a size of the micronuclear genome of approximately 500 Mbp [9] organized in about 120 chromosomes, while the micronuclear genome of the only distantly related oligohymenophorean ciliate *Tetrahymena thermophila* contains about 157 Mbp in five chromosomes [1,10].

The genome of macs, on the other hand, is organized in shorter linear molecules. Each of these short macronuclear molecules carries all sequences necessary for its replication and all are terminated with telomeric sequences of homogeneous length at both ends, but lack centromeric DNA sequences [1]. The DNA complexity of macronuclear genomes is much lower than in their corresponding mics [11]. Most extremely, in stichotrichous ciliates as, for example, *Oxytricha* or *Stylonychia,* up to over 95% of the micronuclear DNA sequences are eliminated during development of a new mac, leaving behind only sequences coding for genes and sequences necessary for their transcription and replication. In stichotrichous ciliates these remaining sequences are organized in 15,000–20,000 different short molecules, with sizes between several hundred base pairs up to more than 20 kbp with an average length of about 2–4 kbp [12,13]. Because of their small size, macronuclear molecules are often called nanochromosomes in these ciliates. Usually they only encode one gene; in *Oxytricha*, only 10% of the nanochromosomes contain sequences coding for more than one gene [13].

In the oligohymenophorean ciliate *Tetrahymena*, a much lower percentage of micronuclear DNA sequences are eliminated during macronuclear development: only about 34% of the micronuclear genome is removed from the macronuclear genome, the remaining sequences are organized in 187 macronuclear molecules with an average length of about 500 kbp [14], always encoding many genes separated by spacer DNA. In these ciliates, a well-conserved 15 nt chromosome breakage site (CBS) has been identified which is necessary and sufficient for fragmentation of micronuclear sequences into macronuclear DNA molecules to occur. After breakage, the CBS and about 20 additional base pairs on both sides are lost [15,16] and telomeric sequences are added de novo to both ends of each fragment. Until now, proteins which recognize this CBS have not been identified, but it has been speculated that a homing endonuclease could have been domesticated to perform fragmentation [17]. In *Tetrahymena*, loss of DNA caused by fragmentation only accounts for a minor amount of sequences eliminated during development of a new mac. In fact, most DNA is eliminated as internal DNA deletion, by removal of specific sequences from internal regions of chromosomes without generating new stable ends (Fig. 7.2A).

Ciliate micronuclear genomes contain numerous internal eliminated sequences (IESs), interrupting macronuclear precursor sequences in the micronuclear genome [18–20]. A 2010 genome sequence study identified more than 10,000 IESs,

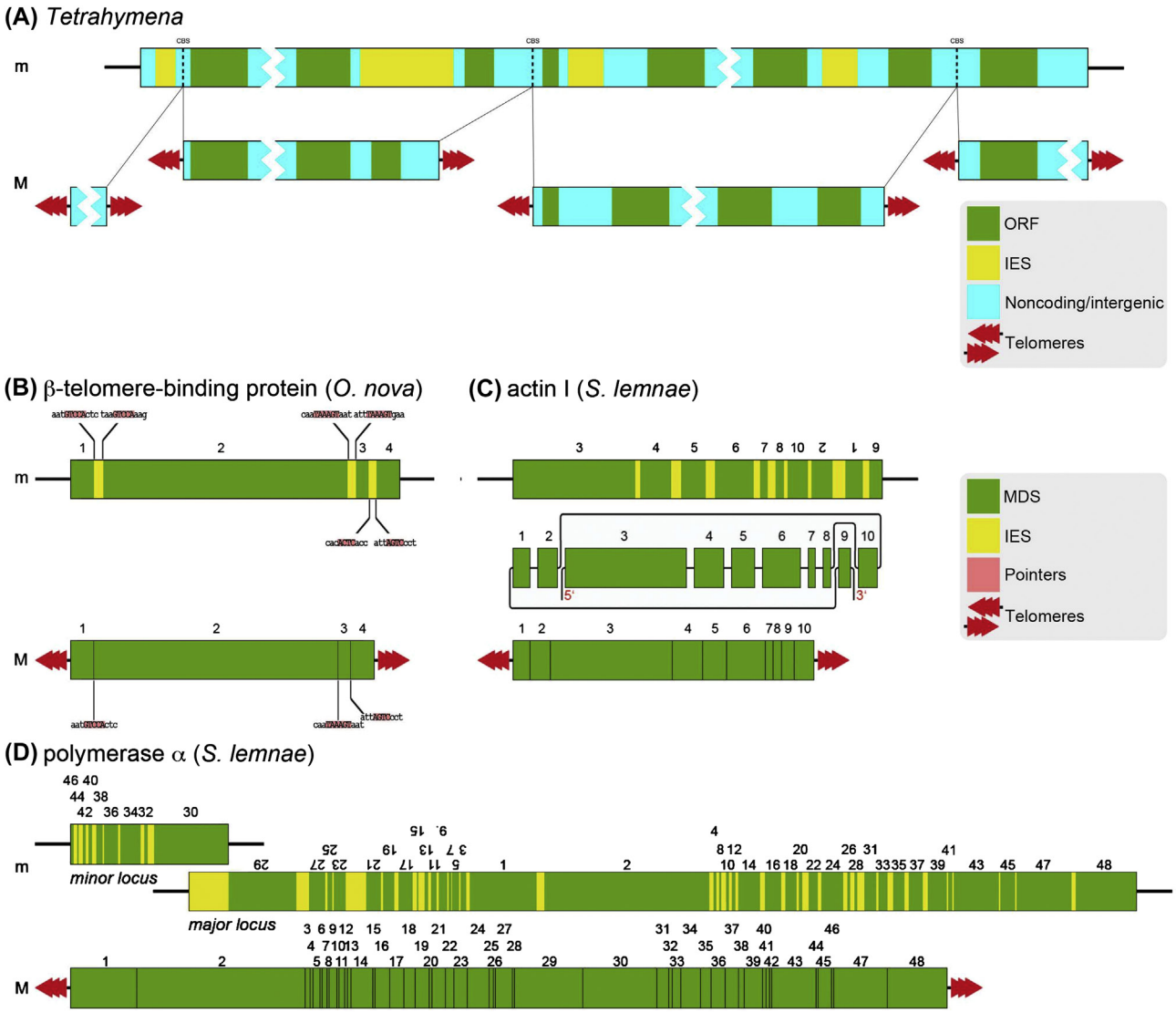

FIGURE 7.2 Examples of the differing micro- and macronuclear genome organization in oligohymenophorean and stichotrichous ciliates. (A) Scheme of the different organization of a micronuclear locus (*m*) and its corresponding macronuclear molecules (*M*) in *Tetrahymena*. Open reading frames (ORF) are shown in green, internal eliminated sequences (IESs) in *yellow*, noncoding, intergenic sequences in *blue*, telomeres as *red arrowheads* and nonspecified flanking sequences as *black lines*. (B–D) Examples of the different organization of genes in the micronuclear (*m*) and the macronuclear (*M*) genome of stichotrichous ciliates. MDSs are shown in green, IESs in *yellow*, pointers in *light red*, telomeres as *red arrowheads* and flanking micronuclear sequences as *black lines*. Numbers show the order of MDSs in the macronuclear nanochromosome, upside down numbers indicate an inverted orientation of the MDS in the micronuclear genome. (B) The linear β-*telomere-binding* gene in *O. nova* [47]. At the borders between MDSs and IESs both copies of individual direct repeats (pointers) are indicated, of which only one copy remains in the macronucleus. (C) The scrambled *actin I* gene in *Stylonychia lemnae* [48]. The rearrangement processes necessary to descramble the micronuclear *actin I* gene into a functional nanochromosome [51] are indicated in the middle scheme. (D) The extensively scrambled *polymerase* α gene is encoded in two different loci in the micronuclear genome of *S. lemnae* [49].

with sizes ranging from several hundred base pairs up to 10 kbp (on average 2–3 kbp), residing in the micronuclear genome of *Tetrahymena*, most, if not all, in noncoding regions [10]; many of these IESs resemble transposable elements and most contain short terminal direct repeats. In the early stages of macronuclear development, before becoming eliminated, these micronuclear-specific sequences adopt heterochromatic chromatin organization: chromatin to become removed has been shown to be under-acetylated [21]. Moreover, di- and tri-methylation at lysine 9 of histone H3 (H3K9me2,3), which are known to be consistent histone modifications assigned to heterochromatin organization in many eukaryotes, as well as tri-methylation at lysine 27 (H3K27me3) appear to be associated to IESs in the developing mac and disappear after elimination has occurred [22]. One of three methyltransferase genes identified in the genome of *Tetrahymena*, *EZL1*, is expressed only during conjugation and seems to be involved in introducing the H3K27me3 modification, which in turn seems to facilitate

methylation of H3K9 [23]. Moreover, a chromodomain protein, Pdd1p, related to the conserved heterochromatin protein 1 (HP1), could be identified to be expressed only during conjugation. It localizes to the developing anlage, where in later stages of conjugation it colocalizes with aggregates of IESs [24]. Gene knockout studies showed that Pdd1p is required for DNA deletion and, when tethered to sequences normally remaining in the mac, caused these sequences to become eliminated [22]. Pdd1p contains two chromodomains and one chromoshadow domain, with the first chromodomain presumably being responsible for DNA targeting, while the other two domains seem to be involved in histone modifications and Pdd1p aggregation [25].

During 2010, two genes, *TPB1* and *TPB2*, were identified which show high similarity to piggyback transposases and lack other transposon features. Differently to other transposons and transposon-like elements residing in the micronuclear genome of *Tetrahymena*, they are not removed from the developing mac, but instead are expressed specifically during conjugation [26]. Namely, Tpb2p seems to be involved in DNA deletion: it colocalizes with Pdd1p forming aggregates, and knockdown strains are deficient in DNA deletion and chromosome fragmentation. When expressed in bacteria, Tpb2p exhibits a weak endonuclease activity. This endonucleolytic activity generates DNA ends resembling the termini of DNA molecules which occur as intermediate products during removal of the M-element, an IES often used as model for deletion studies in *Tetrahymena* [27]. This suggests that Tpb2p could be required for DNA cutting in the process of DNA elimination. During the process of domestication, *TPB2* seems to have acquired further exons in its C-terminus in addition to piggyback domains, leaving it nearly twice as long as most other piggyback transposases. Presumably through these additional C-terminal sequences, including a zinc-finger domain, Tpb2p is now targeted to heterochromatic sequences [28].

Even though IESs in *Tetrahymena* are located in noncoding regions, therefore not necessarily requiring exact excision of sequences, and are marked only imprecisely by adopting heterochromatic chromatin structure, most IESs are nevertheless removed rather precisely from the genome of the developing mac, with only minor occasional junction variations of less than 10 bp [29,30]. Only some IESs are eliminated with junctions varying by several hundred nucleotides [31]. IESs eliminated precisely seem to be flanked by pairs of *cis*-acting sequences, for example, the M-element is flanked on both sides by a 10 bp sequence motive, 45 bp away from each end. This sequence motive seems to be responsible for setting distinct deletion boundaries as without it deletion of the M-element becomes variable. Moreover, new boundaries are induced if the motif is inserted within the M-element [32]. Similar flanking sequence motives have also been identified near other IESs. This led to the speculation, that in *Tetrahymena* precisely excised IESs are subdivided into many families, with each family sharing a specific sequence motive for determining IES elimination boundaries [33]. It has been suggested that these sequence motives flanking the heterochromatic sequences destined to be eliminated could help to target Tpb2p and presumably also other similar nucleases to these heterochromatin boundaries, where they would cut the DNA according to their specific sequence requirements [34].

So far, no distinct CBSs necessary for fragmentation could be identified in stichotrichous ciliates. In *Stylonychia*, a 6 nt sequence localized in the 5′- and 3′-subtelomeric region of the nanochromosome coding for a 1.3 kbp gene of unknown function seems to be necessary for its fragmentation [35]. This putative CBS is very similar to a conserved CBS (E-CBS) identified in the hypotrichous ciliate *Euplotes* [36]. In contrast to *Tetrahymena*, in stichotrichous ciliates micronuclear-specific sequences are eliminated largely as long intergenic DNA stretches, separating the macronuclear precursor sequences from each other. These sequences eliminated as bulk DNA consist in large parts of satellite repeats, transposons, and, of some micronuclear-specific coding sequences [9].

Earlier studies in *Stylonychia* already showed by electron microscopy that in the developing mac at the end of polytenization, a large part of the genome adopts heterochromatic features and is excised as ring-like chromatin structures [37]. This appearance of heterochromatic chromatin in the macronuclear anlage was confirmed by studies on histone modification patterns and chromatin plasticity during macronuclear differentiation [2,38]: very early in macronuclear development, repressive histone modifications typical for the germline mic (eg, H3K27me3) are removed, and instead, the anlagen genome becomes associated with histone modifications, such as histone H3 acetylations, typical for "open" chromatin. Sequences which will be retained in the developing mac stay associated with these histone modifications, while repressive markers (such as H3K9me3 or H3K27me3) are introduced to sequences to become removed. Furthermore, similar to *Tetrahymena*, chromatin-modifying proteins, the heterochromatin-specific *Stylonychia* HP1 homolog Spdd1p and a putative E(z)kmt6-like histone methyl-transferase could be localized in the developing anlage simultaneously to the appearance of repressive markers. During mid-2010s it was proposed that in *Stylonychia*, the fate of specific DNA sequences targeted to become organized into either permissive or repressive chromatin, leading to retention and then respectively elimination, could be determined not only by introducing specific posttranslational histone modifications, but also by a preceding deposition of specific histone H3 variants [39]. In *Stylonychia*, eight different histone H3 variants could be identified; some of these variants were shown to be differentially expressed during macronuclear development and to be specifically targeted by posttranslational modifications.

Besides chromatin modifications acting on histones, a further epigenetic modification, methylation of DNA, could be involved in marking sequences for elimination from the anlagen genome [40]. In stichotrichous ciliates, extensive methylation of cytosines as well as hydroxymethylation has been observed to occur in micronuclear-specific transposons and satellite repeat sequences [41,42].

In addition to sequences removed as bulk DNA in the process of fragmentation, similar to *Tetrahymena*, stichotrichous ciliates contain more than 200,000 IESs in their micronuclear genome [9]. In contrast to *Tetrahymena*, IESs in stichotrichous ciliates are smaller, with sizes generally between only 0 and 100 bp [9,43,44] residing in noncoding as well as coding regions. According to the number of interrupting IESs, macronuclear precursor sequences in the micronuclear genome are separated into several blocks of so-called macronuclear destined sequences (MDSs) (Fig. 7.2B–D). IESs in stichotrichous ciliates always are flanked by "pointer" sequences, direct repeats of 2–20 bp in length, with one of the two pointer copies at the 3′-end of MDS *n* and the second copy of the pointer at the 5′-end of MDS *n* + 1 according to their order in the macronuclear nanochromosome (Fig. 7.2B). IESs with a size of 0 bp therefore just consist of tandem repeats of two pointers in the micronuclear genome. After excision of IESs, only one copy of each pointer can be found at the junction between neighboring MDSs, suggesting a homology directed repair mechanism to be involved in IES elimination [43,45]. In *Oxytricha*, thousands of active transposase genes were identified to reside in the micronuclear genome-encoding proteins which are thought to be involved in the excision of IESs. Each of these transposases would be targeted to its specific pointer sequence and then be responsible for the excision of all IESs flanked by this specific pointer sequence [46]. In general, MDSs in the micronuclear genome of ciliates occur in the same order as in the corresponding macronuclear chromosome of the adult mac, they are linearly arranged (eg, Fig. 7.2B, micronuclear *β-telomere binding protein* locus [47]) but in stichotrichous ciliates, more than 30% of the MDSs are arranged in permuted disorder or inverted orientation in the micronuclear chromosomes [9,45] (eg, Fig. 7.2C, micronuclear *actin I* locus [48]) with MDSs sometimes being even located on different chromosomes (eg, Fig. 7.2D, *polymerase α* locus [49]). In order to form functional nanochromosomes, in these cases not only the IESs have to be excised precisely during macronuclear development, as in linearly arranged MDSs, but furthermore scrambled MDSs have to be reordered into their correct macronuclear order and orientation (Fig. 7.2C) (for review: [45]).

In stichotrichous ciliates, each nanochromosome is amplified to its specific copy number in the course of macronuclear development. These copy numbers vary between a few 100 to up to 10^6 copies [1,6,7]. Copy numbers are generated in two amplification steps. First, as in *Tetrahymena*, early in conjugation, before fragmentation into nanochromosomes takes place, anlagen chromosomes are endoreplicated into polytene chromosomes. Already during this first amplification stage, transposon-like elements as well as the IESs become excised from the anlagen genome [50–52]. After this first amplification stage, fragmentation follows and the intervening micronuclear-specific bulk DNA sequences are eliminated and telomere sequences are added de novo to both ends of each nanochromosome. In a second amplification step, all nanochromosomes become amplified, each to its specific copy number. How this specific amplification could be regulated is discussed later.

In the first part of this chapter the organization of the micro- and macronuclear genome of two different ciliate classes, the oligohymenophorean ciliate *Tetrahymena* and the stichotrichous ciliates *Oxytricha* and *Stylonychia* was described, as well as some of the processes and the proteins identified so far which in the course of macronuclear differentiation are necessary in order to develop a new mac from a micronuclear derivative. But how are sequences selected to become organized into heterochromatin and finally deleted?

In the second part, the actual knowledge on the regulation of these selection processes is discussed.

4. EPIGENETIC REGULATION OF MACRONUCLEAR DEVELOPMENT IN *TETRAHYMENA*

One of several early hints that genomic rearrangement during macronuclear development could be regulated epigenetically by information from the parental mac came from studies of two differing cell lines of *Paramecium*, like *Tetrahymena* belonging to the oligohymenophorean ciliates. These two cell lines contained identical mics but differed in their macronuclear genomes. While one cell line kept a specific IES in its macronuclear genome, the second cell line reproducibly excised this IES during macronuclear development. In progenies of crosses of these two cell lines, it could be shown that the genomic alternative, that is, retaining the IES or eliminating it from the macronuclear chromosome, was maternally inherited. Furthermore, when parental macs in these two cell lines were transformed with fragments of either version of the respective macronuclear chromosome, presence of the IES in the parental mac resulted in retention, while absence resulted in elimination of this IES in the newly developed macronuclear genome of its sexual progeny. This suggested that information from the parental mac is involved in selecting sequences for deletion [53].

In 2002, Mochizuki et al [54]. presented a model, the scanRNA model, explaining regulation of macronuclear development in *Tetrahymena*. This model was based mainly on the identification of a population of small RNA (sRNA) molecules, about

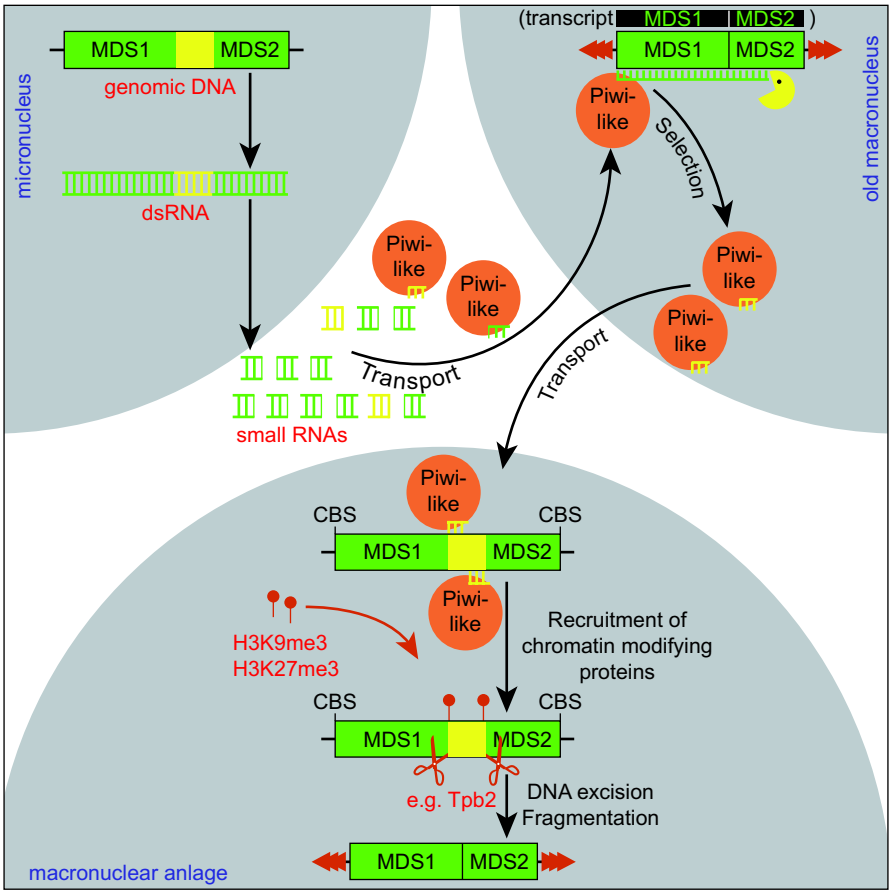

FIGURE 7.3 **The scanRNA model.** sRNAs are processed from long dsRNA molecules deriving from bidirectional transcription of the micronuclear genome. After association to piwi-like proteins, sRNAs invade the parental macronucleus. By interacting with the macronuclear genome [or maybe transcripts of the macronuclear genome (*black*, *in brackets*)], complementary sequences (*green*) are retained in the parental macronucleus and become eventually degraded. sRNAs homologous to micronuclear sequences (*yellow*) remain free to invade the macronuclear anlage. By recruiting chromatin-modifying enzymes to their corresponding sequences in the anlagen genome, sRNAs mark these sequences for deletion by inducing heterochromatin formation. Heterochromatic sequences are targeted by endonucleases, such as Tpb2, a domesticated piggyback transposase, excised and finally degraded. The anlagen chromosomes become fragmented at sites defined by the conserved CBSs and telomeres (*red arrowheads*) are added de novo to each end of the new macronuclear chromosomes. *Modified after Mochizuki K, Fine NA, Fujisawa T, Gorovsky MA. Analysis of a piwi-related gene implicates small RNAs in genome rearrangement in Tetrahymena. Cell 2002;110(6):689–99.*

27 nt in size, appearing in the early stages of macronuclear development and linked regulation of macronuclear development to the mechanism of RNA interference (RNAi) which only shortly before had been described in nematodes [55]. Very early in the course of macronuclear development, appearing sRNAs were shown to be homologous to both kinds of sequences present in the micronuclear genome, to the MDSs as well as the micronuclear-specific sequences. In later stages of macronuclear development, sRNAs homologous to micronuclear-specific sequences become enriched. Furthermore, they identified proteins expressed during these stages of macronuclear development and required for elimination, which were homologous to proteins known to play key roles in RNAi-related pathways in other organisms. In the scanRNA model (Fig. 7.3), very early in macronuclear development, the entire micronuclear genome is transcribed bidirectionally into double-stranded (ds) RNA molecules. These dsRNA molecules are then processed by Dcc1, a dicer-like protein [56,57], into sRNAs, called scan RNAs (scnRNAs), with sizes of about 27 nt. scnRNAs are associated with Twi1, a member of the Piwi family [54], which during conjugation was shown first to appear in the parental mac and in later stages to localize to the developing anlage. According to the model, scnRNAs/Twi1 complexes assemble in the cytoplasm and are then shifted into the parental mac. scnRNAs homologous to macronuclear sequences are retained in the parental mac by their complementary macronuclear DNA sequences (hence the term scnRNA) and become degraded. In contrast, scnRNAs homologous to micronuclear-specific sequences stay free to leave the parental mac and to invade the developing anlage. There they recruit chromatin-modifying enzymes to their complementary DNA sequences, that is, the micronuclear-specific sequences, marking them to be excised by recruiting chromatin-modifying enzymes, organizing these sequences into heterochromatin.

The scnRNA model very conclusively explains the observations obtained in the *Paramecium* experiments mentioned earlier: offspring of ciliates from cell lines which kept the specific IES in their macronuclear genome or of ciliates, into which this IES was artificially introduced in the parental mac, always retained the IES during development of a new mac. According to the model, in these cases, scnRNAs homologous to this IES would be retained in the paternal mac as they would be scanned for by the IES sequence present in the mac. As a consequence, no scnRNA homologous to the IES would reach the developing mac, and therefore this IES would not be marked for excision from its genome.

It should be noted, however, that the 2012 high-throughput sequence analysis of sRNAs from different time points during macronuclear development of *Tetrahymena* [58] showed that the scnRNA model in its original form seem at least to need some modifications. By this sRNA analysis, it could be shown that in contrast to the uniform bidirectional transcription of the entire micronuclear genome proposed in the scnRNA model, early in conjugation about 80% of all 27 nt sRNA could be assigned to only 25% of the micronuclear sequences coding for IESs. Furthermore, sequences to be retained in the mac were transcribed to a much lower extent (only 15% of all sRNAs analyzed) than predicted by the model for these early stages of conjugation. Furthermore, such bias of sRNA transcription could also be seen in mutants of *TWI1*. In these mutants, according to the model, the scanning pathway should be impeded, and therefore no enrichment should be obtained. This argues for the presence of an already highly biased transcription resulting in an enrichment of sRNAs homologous to some IESs sequences, instead of a uniform transcription of the micronuclear genome, followed by a subsequent scanning process of the sRNAs in the paternal mac. But so far mechanisms which could regulate such a biased transcription are unknown. Until now, direct experimental evidences arguing in favor for a scanning mechanism in the parental mac only could be obtained in *Paramecium*. In these ciliates, artificial introduction or deletion of sequences from the parental mac led to retention or elimination of these sequences in the newly developed mac of offspring cells [53,59].

In later stages of macronuclear development, the relative amount of sRNAs from macronuclear precursor sequences decreases significantly as proposed by the model, but whether a scanning process is responsible for this decrease or transcription of micronuclear sequences is already regulated dynamically, still needs to be determined. As one alternative to the original scanRNA model, a two-step pathway resulting in an enrichment of sRNAs homologous to micronuclear-specific sequences has been suggested. A selective transcription of dsRNAs from the micronuclear genome would be the first step to determine a sequence for elimination [60,61]. In a secondary step, a scanning process as suggested in the scanRNA model would than augment this sequence selection. A mechanism how such scanning in the parental mac could be performed still needs to be elucidated. At least for *Paramecium*, it has been proposed that in the parental mac an interaction between invading sRNAs and transcripts of all macronuclear chromosomes instead of the macronuclear genome itself could be responsible for the retention of sRNAs homologous to macronuclear sequences [59,62]. This would then leave sRNAs homologous to micronuclear-specific sequences free to invade the developing anlage and to recruit chromatin-modifying enzymes to their corresponding sequences marking them for elimination, similar as in RNA-induced transcriptional gene silencing in *Schizosaccharomyces pombe* and other organisms [4,63]. By evolving pathways to excise these sequences, elimination can thereby be seen as most extreme form of gene silencing.

5. EPIGENETIC REGULATION OF MACRONUCLEAR DEVELOPMENT IN STICHOTRICHOUS CILIATES

sRNAs with sizes of about 27 nt have also been detected during early stages of macronuclear development in stichotrichous ciliates [38,64,65]. In contrast to the findings in *Tetrahymena*, 2012, in high-throughput sequence analyses of conjugation-specific sRNAs in *Oxytricha*, no micronuclear-specific sRNAs were observed accumulating during macronuclear development, but instead a high level of a macronuclear-specific class of sRNAs were identified appearing solely during early macronuclear development [64,65]. These 27 nt long sRNAs originate from bidirectional transcription of the parental mac. They preferentially cover the open reading frames of all nanochromosomes including macronuclear-specific pointer recombination junctions which are only present in the rearranged macronuclear genome. As in oligohymenophorean ciliates, they are associated with Piwi-like proteins (Mdp1 in *Stylonychia* [66] and Otiwi1, one of 13 Twi1 homologs identified in *Oxytricha* [64]), which as in *Tetrahymena* were shown to first appear in the parental mac and subsequently to localize to the developing macronuclear anlage [2,64,67]. In contrast to the oligohymenophorean scanRNA model, in which micronuclear-specific scnRNAs target sequences for elimination, the macronuclear-specific sRNAs of stichotrichous ciliates seem to mark sequences for retention in the developing mac. Microinjection of synthetic sRNAs complementary to IESs into conjugating cells leading to retention of these IESs and therefore reprogramming their developmental fate [64] confirmed this suggestion. Moreover, their retention was shown to be stably inherited over asexual as well as sexual generations. In stichotrichous ciliates, the macronuclear-specific sRNAs could protect their corresponding sequences in the anlagen genome against introduction of epigenetic marks as DNA or histone methylation. Thereby all macronuclear destined

sequences would stay in an open chromatin state, while all other sequences would be marked for deletion by packaging them into heterochromatin.

Marking sequences by chromatin modifications or protecting sequences by a pool of 27 nt sRNA molecules can only be imprecise. Such imprecise marking may be sufficient for sequences excised as bulk DNA and residing in noncoding sequences; however, IESs, most of them interrupting sequences destined to contribute to coding sequences in the macronuclear genome, need to be excised precisely in order to generate nanochromosomes encoding functional open reading frames. Indeed, so far in mature mac nanochromosomes, junctions between consecutive MDS were always observed to be correct accounting for a precise elimination of IESs in stichotrichous ciliates. But a different picture emerges when looking at intermediate molecules, generated as transient products during the process of genome rearrangement. A surprisingly high percentage of such intermediates show traces of IESs which had been incorrectly excised from their macronuclear precursor DNA [51]. In these cases, either too much or not enough sequences were excised leading to nanochromosomes with either additional or missing sequences. The fact that this does not lead to a high percentage of nonfunctional nanochromosomes in the mature macs suggests that either incorrectly rearranged nanochromosomes are selectively eliminated or that they are corrected by a proofreading mechanism. All cases of imprecise excision observed nevertheless always took place between pairs of direct repeats, called cryptic pointers, in contrast to the original pointers which should have been used for precise excision. Imprecise marking of specific sequences by modifying chromatin structure could explain the observed frequent occurrence of imprecise IES excision in intermediates. Excision could take place by homologous recombination between any direct repeats localized in the vicinity of the endogenous correct pointer repeats using them as cryptic pointers.

A previous experiment had already suggested before that in *Stylonychia* a proofreading mechanism requiring template sequences from the parental macronuclear genome could be involved in macronuclear development of this ciliate. In this experiment, various constructs of a micronuclear locus containing precursor sequences corresponding to an entire macronuclear nanochromosome as well as its IESs and additional flanking micronuclear-specific sequences were injected into anlagen before fragmentation took place [35,68]. Injection of constructs containing deletion of the micronuclear locus, missing up to more than 200 bp of one end of the nanochromosomal precursor sequences, resulted in fragmentation of these sequences from their flanking micronuclear-specific sequences. Surprisingly, after finishing macronuclear development, sequences missing in the injected deletion constructs had been filled up to correct full-length nanochromosomes. This suggested that a proofreading mechanism could exist ensuring that nanochromosomes, which had been truncated after fragmentation from their neighboring micronuclear-specific sequences, are filled up to their correct length. In order to supplement missing sequences correctly, such a proofreading mechanism would require full-length templates of all macronuclear nanochromosomes.

Moreover, as described earlier, in stichotrichous ciliates up to 30% of the genes occur in a scrambled disorder in the micronuclear genome. With pointer sequences between 2 and 20 bp in length, pointers are not specific enough to provide sufficient information to assure correct alignment of consecutive MDSs, especially if MDSs are scrambled in the mic, and therefore can be located far apart or even as in the case of the *polymerase α* in *Stylonychia* on different chromosomes (Fig. 7.2C and D).

In 2003, a theoretical model, the template-guided model of recombination, was proposed by Prescott et al. [69] explaining how processes necessary to rearrange the micronuclear genome into the genome of the mature mac could be regulated in stichotrichous ciliates (Fig. 7.4). They suggested that templates containing all sequences of the macronuclear genome are produced from the parental mac and transported into the early macronuclear anlage. In the anlage, templates align to their homologous DNA sequences of the early anlagen genome, thereby bringing corresponding direct pointer repeats into very close vicinity, while intervening micronuclear-specific sequences extrude as loops from the DNA–template complexes. Homologous recombination between the aligned pointer sequences then would allow excision of the loops containing the micronuclear-specific sequences, leaving behind only one copy of the pointers in the mature macronuclear genome.

In 2008, strong experimental support was provided that indeed in stichotrichous ciliates, DNA processing during macronuclear development could be guided by templates, presumably RNA molecules, originating from transcription of the parental mac [70]: degrading putative RNA templates of specific macronuclear nanochromosomes by applying RNAi techniques during macronuclear development resulted in aberrant gene unscrambling of the corresponding micronuclear loci. Furthermore, when early in macronuclear development, artificial RNA template molecules were injected, in which the template sequences corresponding to MDSs of a specific macronuclear nanochromosome were lined up in a different order to the MDS order of the endogenous template molecules, these artificial templates led to reprogrammed DNA rearrangements resulting in nanochromosomes with MDSs arranged according to the order of the artificial template.

The template-guided model conclusively can explain how rearrangement processes, necessary for reordering of scrambled genes into their corresponding macronuclear nanochromosomes as well as correcting imprecise excision of IESs and filling up truncated nanochromosomes after fragmentation, could be regulated. But why such imprecise IES excision

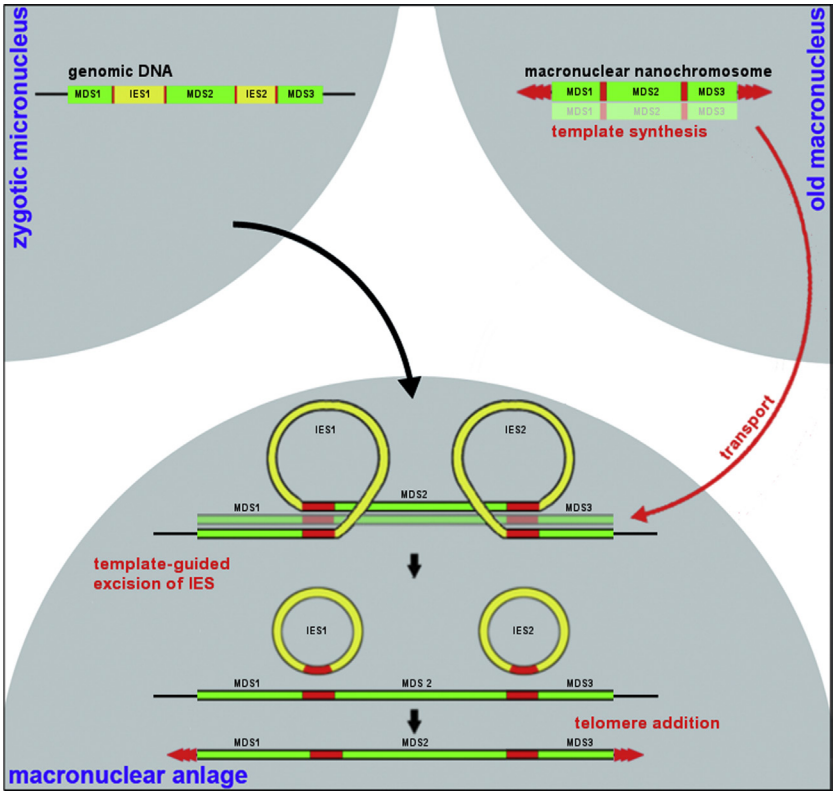

FIGURE 7.4 **The template guided model.** Genome rearrangement processes are guided by templates (*faint green* and *red*) of all nanochromosomal sequences. These templates derive from transcription of all nanochromosomes in the old (parental) macronucleus. Templates are transported into the macronuclear anlage and align to their corresponding sequences of the anlagen genome, thereby aligning both pointer repeats (*red*) at the junction of consecutive blocs of macronuclear destined sequences (MDSs, *green*). IESs (in *yellow*) looping out as pointers align, are excised by homologous recombination between the two pointer repeats. Finally, fragmentation into nanochromosomes occurs at the ends of template covered regions and telomeres are added de novo to each end of the newly arranged nanochromosome *(Modified after Prescott DM, Ehrenfeucht A, Rozenberg G. Template-guided recombination for IES elimination and unscrambling of genes in stichotrichous ciliates. J Theor Biol 2003;222(3):323–30.)*. In this scheme, descrambling, arranging scrambled MDS according to their order in the correct nanochromosomal order is not shown. Fig. 7.2C gives a hint on how sequences of a scrambled micronuclear gene would need to bend and twist in order to unscramble by aligning to its nanochromosomal template. (More information on the gymnastics of DNA processing in ciliates can be found in Ref. [45].)

should occur is difficult to explain. In the template-guided model, the macronuclear precursor sequences should align precisely according to the template sequences. If imprecise IES excision should take place, correction by a proofreading mechanism should occur concomitantly with the excision process, while the template molecule is still aligned, not leading to imprecise intermediate molecules detectable by PCR methods.

Therefore, for stichotrichous ciliates, a third model was suggested combining aspects of both the scanRNA model as well as the template-guided model of recombination [71]. This model was modified according to the finding that in these ciliates, sRNAs originate from the parental mac targeting sequences for retention in the developing mac [64], as discussed earlier. In this model (Fig. 7.5), MDSs in the early anlagen genome are marked by homologous sRNAs which originate from bidirectional transcription of the parental macronuclear genome early during conjugation. After processing of the dsRNA molecules into sRNAs, they are associated to Piwi-like homologs and subsequently invade the developing mac. In the developing mac, these sRNAs protect their corresponding sequences of the anlagen genome from being marked for excision by chromatin-modifying enzymes [64,65]. Excision of IESs takes place by homologous recombination between direct repeats located between MDSs protected by sRNAs. As marking of sequences by chromatin modification is very imprecise, not only the correct pointer repeats, but also cryptic pointers, random direct repeats located near the correct pointer sequences, can be used as sites for excision by homologous recombination, thereby leading to imprecise excision of IESs. Later during conjugation all nanochromosomes of the parental mac are transcribed into long RNA molecules, presumably also covering the telomeric ends, which then migrate into the developing anlage too. There, these RNA molecules guide the alignment of MDSs according to their order in the parental nanochromosomes. Furthermore, they serve as templates for a proofreading mechanism repairing imprecise excision and filling up

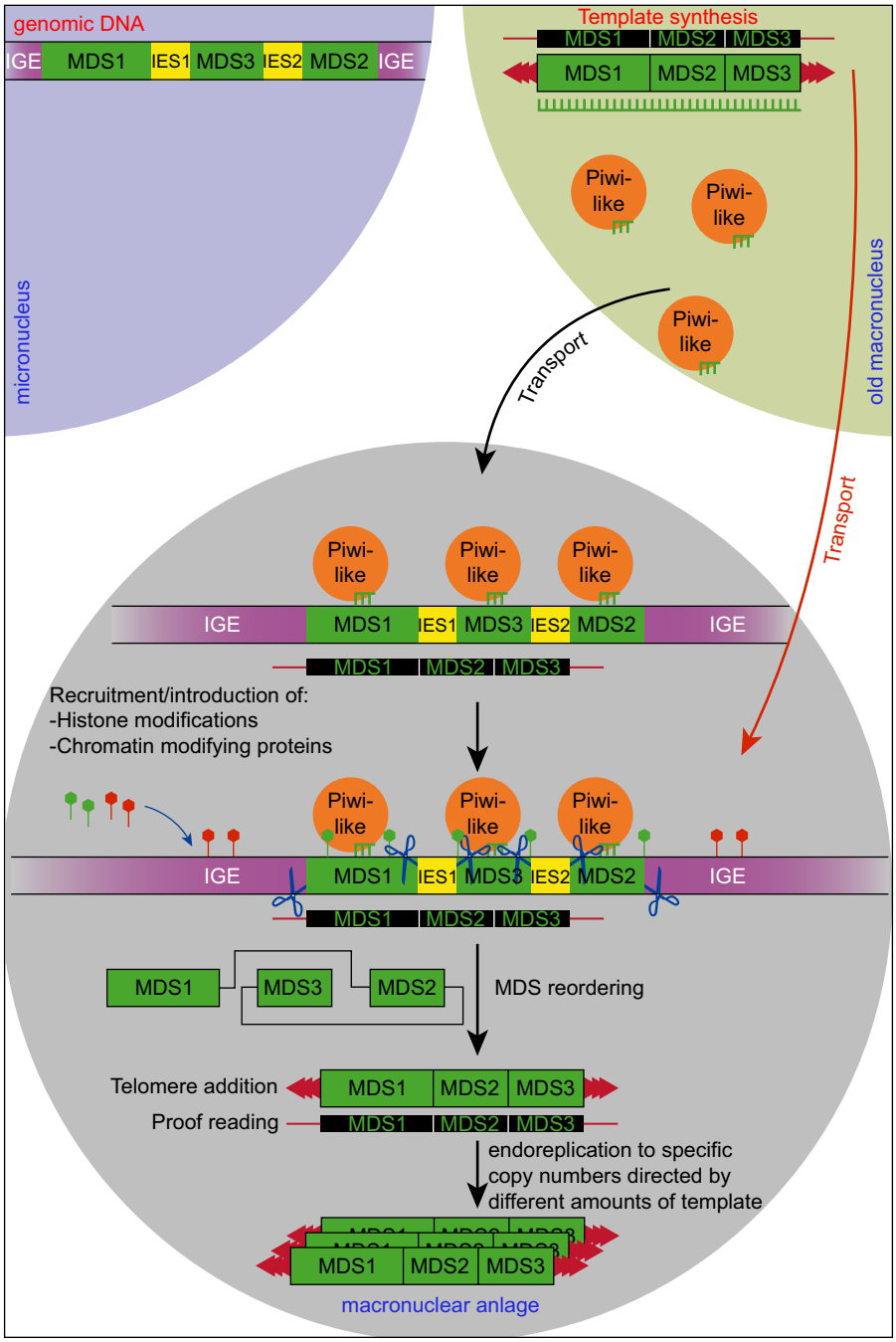

FIGURE 7.5 **Model explaining macronuclear development in stichotrichous ciliates.** Early in macronuclear development, bidirectional transcripts of the old (parental) macronucleus are processed into sRNAs (green) and associated to piwi-like proteins. These complexes invade the early macronuclear anlage and mark corresponding sequences of the anlagen genome as macronucleus destined sequences (*green*). Chromatin of all other sequences [intergenic sequences (IGE, *purple*) and IESs (*yellow*)] is modified by chromatin-modifying enzymes and thereby marked for deletion. Excision of IESs takes place (possibly by not yet characterized different tranposases) between the two pointer repeats of consecutive MDSs or any direct repeats in their vicinity. In addition to the early transcription of the old macronucleus, generating the sRNA molecules, the old macronucleus is further transcribed into the long probably full length transcripts (*black*) including the telomeres. They are transported into the anlage and align to corresponding sequences of the anlagen genome thereby guiding the reordering of scrambled MDSs. Furthermore, they act as templates for correcting imprecise excision of IESs or for complementing nanochromosomes which became truncated by fragmentation. Telomeres are added de novo to each nanochromosomal end. Finally, by a still unknown mechanism, the amount of template molecules, which reflects the copy number of each nanochromosome in the old macronucleus, then determines the level of amplification of each nanochromosome, thereby defining the specific copy number of each nanochromosome in the mature new macronucleus. *Modified after Fang W, Wang X, Bracht JR, Nowacki M, Landweber LF. Piwi-interacting RNAs protect DNA against loss during Oxytricha genome rearrangement. Cell 2012;151(6):1243–55; Juranek SA, Lipps HJ. New insights into the macronuclear development in ciliates. Int Rev Cytol 2007;262:219–51.*

missing subtelomeric sequences of macronuclear nanochromosomes after fragmentation. How this proofreading could be performed still needs to be determined.

Finally, RNA templates from the parental mac are not only involved in guiding DNA rearrangement processes during macronuclear development, they also seem to play an important role in regulating the amplification of nanochromosomes to their specific copy number. When the number of specific templates during macronuclear development is changed experimentally, either by applying RNAi techniques to decrease the amount of a specific endogenous template or by injecting artificial RNA template molecules to increase the amount of a specific template, the copy number of the corresponding nanochromosome in the adult macs is changed according to the experiment applied [6,7].

6. CONCLUSION

At first sight, the contrast in RNA regulation mechanisms between Oligohymenophorean and Stichotrichs seems to be surprising: sRNAs originate from different nuclei (mic versus parental mac), they target sequences inducing opposing developmental fates (deletion versus retention) and, in the case of Stichotrichs, an additional RNA-regulated mechanism is required to guide genome reordering and proofreading. But both ciliate classes are only very distantly related, their lineages separating over a billion years ago comparable to the linages of humans and fungi [72,73]. Moreover, this variability in the regulation of genome rearrangement processes in both ciliate classes show the high adaptability of RNA-induced regulation in genome dynamics making ciliates very useful model systems to study pathway regulated by ncRNA molecules. Regulation of genome dynamics depending on ncRNAs have shown to be widespread in eukaryotes. Ciliates, extensively using variations of these regulation mechanisms, provide excellent model systems to study the pathways involved in these processes.

GLOSSARY

Anlage During development of a new macronucleus from a derivative of the micronucleus, the developing macronucleus is called anlage.

Cryptic pointers Direct repeats in the vicinity of the correct pointer repeats. Use of cryptic pointers for IES elimination leads to imprecise elimination: These mistakes have to be corrected in order to generate functional macronuclear nanochromosomes.

Endoreplication DNA replication without nuclear division.

Macronucleus Somatic nucleus of ciliates.

Micronucleus Germline nucleus of ciliates.

Nanochromosome Short DNA molecule in the stichotrichous macronucleus, terminated by telomeric sequences and containing all sequences necessary for its replication, mostly encoding only one gene and all sequences necessary for its transcription.

Pointer repeats Direct repeats (between 2 and 20 bp) flanking IESs in the micronuclear genome. One copy of the direct pointer repeat is localized at the 3′ end of MDS n, the second pointer repeat is localized at the 5′ end of MDS $n+1$. After excision of IESs, only one copy of the pointer repeats remains at the junction of consecutive MDSs in the macronuclear nanochromosome.

LIST OF ABBREVIATIONS

CBS Chromosome breakage site
IES Internal eliminated sequence
Mac Macronucleus
MDS Macronucleus destined sequence
Mic Micronucleus
scnRNA Scan RNA

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