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RNA-Mediated Epigenetic Programming of Genome Rearrangements

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Abstract

RNA, normally thought of as a conduit in gene expression, has a novel mode of action in ciliated protozoa. Maternal RNA templates provide both an organizing guide for DNA rearrangements and a template that can transport somatic mutations to the next generation. This opportunity for RNA-mediated genome rearrangement and DNA repair is profound in the ciliate *Oxytricha*, which deletes 95% of its germline genome during development in a process that severely fragments its chromosomes and then sorts and reorders the hundreds of thousands of pieces remaining. *Oxytricha*'s somatic nuclear genome is therefore an epigenome formed through RNA templates and signals arising from the previous generation. Furthermore, this mechanism of RNA-mediated epigenetic inheritance can function across multiple generations, and the discovery of maternal template RNA molecules has revealed new biological roles for RNA and has hinted at the power of RNA molecules to sculpt genomic information in cells.

Keywords

noncoding RNA; maternal inheritance; Lamarckian inheritance; scrambled genes; ciliates; *Oxytricha*

INTRODUCTION

Protists have surprised molecular biologists with a bewildering array of genetic architectures and mechanisms, including the Nobel Prize-winning discoveries of self-splicing RNA (61) and telomere structure and telomerase (38) in the ciliate *Tetrahymena*, RNA editing in trypanosomes (11) and gene-sized chromosomes, scrambled genes, and programmed deletion of most of their genome in stichotrichous (formerly hypotrichous) ciliates (109). Therefore, these species offer model systems for the study of programmed DNA rearrangements on a large scale.

Scrambled Genomes

Scrambled genes exist in many eukaryotes, ranging from split ribosomal RNAs in several taxa, including *Chlamydomonas* (24), *Euglena* (128), *Plasmodium* (34), *Theileria* (53), and even *Diptera* (107), to nuclear transfer RNA genes in the red alga *Cyanidioschyzon merolae*

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(64, 125), the split *bursicon* locus in mosquitoes (118), two recent examples in humans (15, 72), and the mitochondrial genome of the flagellated protist *Diplonema papillatum* (82). Rarely, however, are the gene fragments actually sewn back together at the level of DNA to create a contiguous gene from all pieces. Notable exceptions include *V(D)J* splicing and class-switch recombination in the vertebrate immune system (81). *Trans*-splicing of RNA molecules (79), and even of proteins mediated by inteins (132), provides another mechanism to merge functional segments located on disparate parts of a genome.

DNA elimination is the most common type of genome rearrangement that occurs during development in a variety of eukaryotes. Chromatin diminution in somatic cells of the parasitic nematodes *Ascaris* and *Parascaris*, for instance, eliminates approximately 80% of the genome (58, 94). Another example is the programmed loss of millions of base pairs of DNA from lamprey in somatic tissues (123). Programmed DNA elimination can entail the excision of DNA segments with rejoining of flanking sequences or even the loss of entire chromosomes, as in sciarid flies (35).

Examples of programmed DNA rearrangements in organisms other than eukaryotes include DNA-inversion systems that regulate expression of flagellar proteins in *Salmonella* (136) and tail-fiber genes in bacteriophage Mu (40). In many programmed DNA rearrangement systems in bacteria and eukaryotes, the proteins that mediate rearrangement are related to proteins encoded by transposons or viruses. These observations have given rise to suggestions that the DNA rearrangement systems evolved from mobile genetic elements (105), like *V(D)J* rearrangements (131), and several independent events of transposon domestication or recruitment in different lineages of ciliates (9, 21, 101). Although developmentally regulated DNA rearrangements occur in a variety of eukaryotes, they occur at the most exaggerated level in ciliates. In stichotrichous ciliates, such as *Oxytricha* [also called *Sterkiella* (12)] or *Stylonychia*, the changes are truly impressive, causing elimination of 95%–98% of the germline and a shattering of germline chromosomes to produce tiny gene-sized DNA molecules. In the *DNA polymerase α* gene of *Oxytricha trifallax*, for example, a single gene is present in 48 different germline segments whose order and direction are discontinuous with the functional coding sequence (45).

The complexity of DNA-splicing events that take place in stichotrichs makes this system one of the most interesting and challenging cases to study. Moreover, frequent DNA deletions and other extensive genome rearrangements are increasingly associated with several human cancers (13, 14, 16, 28, 72, 76, 129), which underscores the importance of studying such biological events. Nonallelic homologous recombination between short repeated sequences can also produce DNA deletions, duplications, translocations, and recurrent inversions in human somatic cells, even in healthy individuals (30). This observation led to the suggestion that pervasive DNA rearrangements occur in the human genome (30).

Ciliate Nuclear Dimorphism and DNA Compaction

Ciliates are unique in that they possess two different types of specialized nuclei per cell: a compact, archival, germline micronucleus (MIC) and a physically larger somatic macronucleus (MAC) that specializes in gene expression (Figure 1). Species such as *Oxytricha*, the model system in our laboratory, are further distinguished by their striking ability to eliminate almost all noncoding DNA from their genomes during the production of the somatic nucleus. Such wholesale DNA deletion offers a truly elegant solution to the problem of managing so much “junk” DNA: *Oxytricha* simply discards the 95% of its germline genome that is not needed for vegetative growth, then amplifies and repairs the 5% that remains. In many cases, the repair steps involve sorting the remaining DNA sequences through gene unscrambling. This process involves shuffling and linking together hundreds

of thousands of short pieces of DNA to produce over 20,000 distinct genes, many of which are woven together from short segments dispersed over different locations in the encrypted germline genome. Some of these segments may derive from genetically unlinked loci (4).

Whereas the MAC contributes most gene expression during vegetative growth, the functionally inert MIC contributes only to sexual reproduction. The MAC develops from a copy of the diploid MIC after sexual reproduction, during the process of nuclear differentiation. The genomic copies of most MIC genes in stichotrichous ciliates, as well as in the oligohymenophoran *Paramecium*, are crippled by the presence of intervening short, single-copy non-coding DNA sequence elements termed internal eliminated sequences (IESs) (Figure 2). These sequences represent a fraction of the MIC genome that is eliminated. Precise deletion of all IESs is an important part of macronuclear development and is essential for the production of a functional copy of the MAC genome, because IESs frequently interrupt protein-coding regions in species such as *Paramecium* and *Oxytricha*. Extrapolation from the set of MIC genes sequenced to date yields an estimate of more than 150,000 IESs in a haploid germline genome of *Oxytricha* (114) and, hence, over 100,000 recombination junctions that must properly form during MAC development. A fraction of these recombination junctions require MAC-destined sequence (MDS) reordering or unscrambling (see below), whereas the rest are more simple DNA deletions, as in *Paramecium*. On an evolutionary scale, IESs may be the offspring of transposable elements that eventually lost their own transposase activity and now rely on *trans*-acting factors for their excision (57, 116). Recent research in our lab indicates that thousands of active germline transposase genes still participate in the process of IES removal, as well as in the programmed deletion of all transposons themselves (101). This results in the complete absence of both classes of DNA from the mature MAC genome.

The total amount of DNA deleted from the MIC varies from 10% to 20% in *Tetrahymena* to a whopping 85% to 98% (109), or nearly the entire genome, in the spirotrichous ciliates *Euplotes*, *Stylonychia*, and *Oxytricha*. In addition, most MAC chromosomes in spirotrichs and a few other ciliate classes (117) are organized as gene-sized nanochromosomes that average only ~2.2 kb. These nanochromosomes have 20-bp telomeres protecting both ends and very little noncoding DNA; typical 5' leader and 3' trailer sequences average a mere 100 bp each (17). This observation demonstrates that these organisms are model systems in which to study the relationship between noncoding sequence motifs and gene expression. In essence, the execution of this extreme sequence-compaction algorithm during macronuclear development is a natural gene finder (52) because it identifies (and repairs) all RNA- or protein-coding regions necessary for daily growth and discards nearly all of the noncoding DNA.

DNA Unscrambling

One of the greatest surprises in stichotrichous ciliates was the discovery in David Prescott's laboratory (39) that the retained DNA segments (MDSs) that constitute the lucky minority of the genome that is not deleted may be present in a permuted order in the MIC relative to their linear order in the expressed version in the MAC. In addition to the presence of MDSs in the wrong functional order, the system can also tolerate and repair inverted MDSs when a MIC-coding region reads backward relative to the orientation of the flanking MDSs. The maturation of all these MDSs, which represent 2%–15% of the MIC genome in different stichotrich species, requires extensive reordering or inversion of hundreds of thousands of MDSs, in addition to removal of the IESs or intergenic DNA between them, in order to produce functional gene copies in the MAC. For example, the *DNA polymerase α* gene in *O. trifallax* (45) and *S. lemnae* (66) is scrambled into approximately 50 permuted pieces in the MIC; many pieces are in the opposite coding orientation, and some are located on genetically unlinked loci (4). Thus, this gene displays no particular strand asymmetry in the

location of coding regions, which represents a radical departure from the typical textbook gene map. However, some scrambled genes are much less complicated (see, e.g., 20). The process of simultaneously descrambling thousands of genes during development (93) therefore presents a complicated cellular computational problem (63).

Homologous recombination between pairs of identical short sequences, known as pointers, at consecutive MDS–IES junctions simultaneously removes IESs and either reorders or inverts scrambled MDSs. In other words, the DNA sequence present at the junction between MDS n and the downstream IES is generally a precise copy of the sequence between MDS $n + 1$ and its upstream IES, which leads to correct recombination between MDS n and $n + 1$, even over a distance. However, the miniscule length of some pointers [as short as 2 nt, with an average of 4 bp between nonscrambled MDSs and 9 bp between scrambled MDSs (111); current data agree with these estimates] suggests that although they are necessary, pointers are insufficient to direct accurate splicing. Therefore, pointer repeats probably satisfy more of a structural requirement for MDS splicing and play a less significant role in recognition. This finding led Prescott et al. (112) and Angeleska et al. (3) to propose, and Nowacki et al. (103) to test, an alternative epigenetic model for guiding genome unscrambling.

Epigenetics

The differentiation of metazoan cells depends on fundamentally non-Mendelian mechanisms to permit a zygote to develop into various cell types in a highly reproducible fashion. Beyond genetics, an additional level of information that regulates developmental functions has given rise to the concept of epigenetics, the field that embraces all heritable phenotypic characters that lack a genetic basis in the genomic DNA sequence itself. Epigenetic processes are therefore key to cellular differentiation and to the maintenance of stable gene expression profiles during development. Whereas ciliate development does not require cellular differentiation, because most species are unicellular, it does require differentiation of a duplicate germline MIC into a somatic MAC, analogous to the germline–soma distinction in metazoa. Thus, opportunities abound for epigenetic control during these developmental events, particularly during the elaborate cascade of genome-wide DNA rearrangements that operate via physical comparison between germline and somatic genomes in an extraordinarily massive and precise manner.

EARLY HISTORY

Ciliates have enjoyed nearly two centuries of study (122)—first as a group of protozoa, or so-called early animals, and then as a preferred model for many basic biological phenomena—particularly because of their large size (in some cases, more than 100 μm) and transparency. In the nineteenth century, ciliates, along with flagellates and other aquatic microbial eukaryotes, were the subject of extensive studies that focused on anatomy, cortical and ciliary structure, and reproduction (22, 55). Currently, these microbial eukaryotes provide model systems for the study of several problems in genetics, molecular biology, and biochemistry, such as genome organization, cellular structure, endo- and exocytosis, motility, ecology, and evolution. The impact of ciliate research on modern biology has been quite strong throughout the years. In addition to catalytic RNA (61), telomere structure and telomerase (38), and the recent crystal structure of the eukaryotic ribosome (115), studies of ciliates have contributed many fundamental discoveries, including genome-wide DNA rearrangements and numerous epigenetic phenomena (10, 18, 26, 33, 84, 120), particularly because the external cell membranes in the two exconjugant daughter cells are actually the same as the parents' (reviewed in 102).

Maternal Homology-Dependent Effects in *Paramecium* and *Tetrahymena*

The epigenetic influence of maternal cells on the development of their progeny, collectively termed maternal effects, has long been studied in ciliates. *Paramecium*, for example, demonstrates non-Mendelian inheritance of developmental alternatives, such as mating-type determination. In *Paramecium tetraurelia*, mating-type determination occurs during the differentiation of the MAC from a zygotic nucleus, which is genetically competent for the expression of either of the two different mating types, O and E. Notably, even an entirely homozygous micronuclear genome can give rise to a cell of either mating type, which indicates that mating-type determination is not an intrinsic property encoded in the cell's genome. Following nuclear differentiation, the new MAC can express only mating type O or E. However, new macronuclei developing in the cytoplasm of the O parent almost always express O, whereas those developing in the E parent cell almost always express E (96). Numerous experiments by Sonneborn (126) have shown that the parental MAC programs the progeny cell to express the same mating type as the parent, thereby demonstrating a cytoplasmic form of maternal inheritance (Figure 3).

Although the molecular basis for mating-type determination is still unknown, the discovery of homology-dependent maternal effects during DNA rearrangement has shed some light on the molecular basis of epigenetic programming in ciliates. *Paramecium* and *Tetrahymena* provided model systems for the original studies. In particular, *Paramecium* displays two types of developmentally regulated genome rearrangements, precise IES excision and imprecise DNA deletion, both of which can be controlled by homology-dependent maternal effects.

Experiments in the d48 cell line in *Paramecium* provided one of the first discoveries of epigenetic regulation of DNA elimination. Even though these cells do not carry any germline mutations, they are unable to express the surface antigen A because of the complete absence of the *A* gene in the MAC, despite its presence in the MIC. This defect follows a cytoplasmic, non-Mendelian pattern of inheritance (29). Subsequent studies revealed that the only factor determining the absence of the *A* gene in the new MAC is the absence of the same sequence in the parental MAC (56, 134, 135). Introduction of the *A* gene into the parental MAC by microinjection programs its retention in the progeny's somatic genome. Similar experiments demonstrated that excision of a subset of *Paramecium* IESs could be blocked by the presence of an identical IES sequence in the parental MAC, which leads to the specific maintenance of this IES in the somatic genome of sexual progeny for at least two sexual divisions (26). If the presence of an IES does not impede the function of an essential gene, then such experiments can generate so-called IES⁺ cell lines in which the IES has become a permanent part of the somatic genome (Figure 4). However, in *Paramecium*, excision of only a subset of IESs (one-third of those tested) can be blocked in this fashion (27). Such IESs have been termed maternally controlled IESs. To summarize these epigenetic maternal effects: Whether a DNA sequence is maintained in or deleted from the new MAC is determined by its presence or absence in the parental MAC. These observations suggest that the developing MAC must somehow reproduce the rearrangements present in the old MAC, which implicates a transnuclear comparison between genomes.

Similar experiments have been performed in *Tetrahymena thermophila* (18). Transformation of the parental MAC with either of two IESs, known as the *M* and *R* elements, prevents excision of the homologous IESs from the macronuclear genome of the sexual progeny of transformed cells. These experiments led to specific inhibition, even though the presence of the *M* element in the parental MAC also had a slight inhibitory effect on excision of the *R* element in the developing MAC. The presence of the IES-containing construct in the parental MAC also appeared to increase the frequency of aberrant excision events, which rarely occur in nontransformed cells. As in *Paramecium*, in *Tetrahymena* the effect of IES

retention, once induced, can stably pass on to subsequent sexual generations. Furthermore, the presence of the IES sequence alone is sufficient to confer the inhibitory effect, whereas flanking macronuclear sequences alone have no effect at all (18).

A curious difference between these *Tetrahymena* and *Paramecium* experiments is that the sexual progeny of both *Tetrahymena* exconjugants maintain the IES in their MAC, whereas the IES⁺ and IES⁻ characters show distinctly maternal inheritance in *Paramecium*. This observation can be explained by the extensive cytoplasmic mixing that occurs during conjugation in *Tetrahymena* but not in *Paramecium*, and it provides further evidence for cytoplasmic mediation of the influence of the maternal macronuclear genome on the developing MAC.

Many additional features of IESs differ between *Paramecium* and *Tetrahymena*. For example, *Tetrahymena* IESs are much longer and less abundant and are flanked by short direct repeats that can vary in sequence, compared with those of *Paramecium*. Moreover, IES excision in *Tetrahymena* frequently uses discrete sets of alternative boundaries, due to imprecise DNA cleavage on one or both sides of IESs (6, 121). Presumably, this fact, coupled to the greater imprecision of *Tetrahymena* IES elimination (see below), explains why IESs never appear to interrupt coding sequences in *Tetrahymena*. Nonetheless, the homology-dependent maternal effects that lead to examples of specific IES retention in these two species are remarkably similar. This suggests conservation of an ancient mechanism that may also extend to other ciliates, given that the oligohymenophorans *Tetrahymena* and *Paramecium* diverged from the spirotrichous ciliates over one billion years ago. Spirotrich IESs can evolve very rapidly (25, 92).

Early Models for Epigenetic Programming of DNA Rearrangements

Duharcourt et al. (26) proposed the earliest model for epigenetic programming of DNA rearrangements. This model assumed transcription of the whole parental MAC genome, followed by export of the transcripts to the developing MAC, where they regulate rearrangements in a homology-dependent manner. These authors specifically envisioned two possibilities to regulate IES excision: Either the maternal transcripts simply inhibit the excision of sequences with high similarity, or the maternal transcripts provide templates for repair after constitutive elimination (26). The latter idea was rejected after the authors showed that mutations in the sequence injected into the old MAC did not transfer to the IES in the new MAC; this finding was confirmed by the absence of IES cleavage in the IES⁺ cell line (37), where the IES is permanently present in the MAC. However, there is some evidence in support of a protective role for parental MAC transcripts in *Paramecium*: The destruction of specific RNA transcripts by RNA interference (RNAi) leads to the elimination of homologous DNA sequences in the new MAC for both genes (33) and IESs (70). However, a template role for maternal transcripts remains an attractive model to explain the specificity of rearrangements in other ciliates, such as the stichotrichous ciliate *Oxytricha*, in which the macronuclear DNA segments that remain after IES excision are present in a different order relative to their position in the MIC. Maturation of *Oxytricha*'s macronuclear genome requires extensive sequence reordering, or descrambling, in addition to the removal of intervening IESs to construct functional, transcriptionally active genes in the MAC. In this case, the maternal transcripts could provide templates for DNA cleavage, ligation, and repair during the DNA-descrambling process (112).

MACRONUCLEAR DEVELOPMENT

It is a particularly salient feature of ciliate biology that germline and somatic functions are ensured by two different kinds of nuclei: the diploid MIC and the DNA-rich MAC. The two kinds of nuclei differ tremendously in terms of not only their appearance but also their

function. Whereas the large MAC is transcription-ally active and governs a cell's gene expression (somatic function), the MIC hosts the germline genome that usually does not contribute to messenger RNA (mRNA) production but transmits the DNA to sexual progeny (germline function). Meiosis of the MIC initiates the sexual cycle, and new micro- and macronuclei develop from the zygotic nucleus after fertilization; the old MAC eventually degrades and is replaced by the new one (Figure 1).

In most ciliates, not only is the genome amplified to a high ploidy level during macronuclear development, but large-scale genome rearrangements must also occur. Such extensive DNA processing is essential for the formation of the functional somatic genome and occurs in a highly reproducible manner from one sexual generation to the next. The rearrangement takes place at a specific time period following some initial amplification of the diploid genome but before the final ploidy level is reached. Genome rearrangement in most well-studied ciliate species entails only sequence elimination, including transposable elements, IESs, and repeated sequences; however, several spirotrich species (46), as well as *Chilodonella*, a representative of the class Phyllopharyngea (54), also have the added layer of scrambled genes that require MDS reordering between the MAC and the MIC. Estimated IES density, depending on the species, ranges from 6,000 in *Tetrahymena* (133) to approximately 60,000 in *Paramecium* (27) to roughly 200,000 in *Oxytricha* (25)—order-of-magnitude estimates for the number of unique IES copies per haploid genome. Furthermore, roughly one-third of the IESs in *Oxytricha* are estimated to be scrambled in the MIC; that is, they lie between discontinuous MDS segments.

DNA-elimination events in ciliates can be either imprecise or precise, with notable differences between species (Figure 2). Imprecise elimination of repetitive germline sequences (transposons, transposon-like elements, and minisatellites) can either result in the fragmentation of germline chromosomes into shorter macronuclear chromosomes, with telomere addition occurring at variable ends, or lead to the imprecise rejoining of flanking sequences (67). In the latter case, heterogeneous internal deletions can extend a few thousand base pairs and can occur between variable short direct repeats that contain at least one 5'-TA-3' dinucleotide. Precise DNA elimination, however, refers to the excision of short IESs (in species such as *Paramecium* or *Oxytricha*) or some transposable elements (in *Tetrahymena*) that interrupt open reading frames but can also be found in non-coding regions, including introns (106). Their precise excision, especially those located within coding regions, is required for the construction of a functional somatic genome.

Small RNAs in *Paramecium* and *Tetrahymena*

Numerous recent studies have revealed the presence of two distinct RNAi pathways that are involved in mediating various types of homology-dependent effects in ciliates. Post-transcriptional gene silencing can be induced in *Paramecium* by truncated or untranslatable transgenes (31, 120) or by feeding cells with *Escherichia coli* that express double-stranded RNA (dsRNA) (32). In each case, silencing of the targeted genes correlates with the accumulation of homologous ~23-nt silencing RNAs (siRNAs) (33, 104). The same siRNAs are also involved in mRNA silencing when *Paramecium* cells are fed with *E. coli* that express long dsRNA corresponding to a target gene. In *Tetrahymena*, a limited number of loci produce a class of ~23–24-nt siRNAs (68). Experimentally induced hairpin transcripts are processed into siRNAs and trigger the silencing of homologous genes (47). In both *Tetrahymena* and *Paramecium*, one of the Dicer-related proteins is involved in the biogenesis of siRNAs (69, 71). However, the 23-nt siRNAs cannot explain other epigenetic effects, such as the maternal inheritance of alternative DNA rearrangements that do not depend on experimental silencing.

A second RNAi pathway, which has been described mostly in *Tetrahymena thermophila*, is restricted to the sexual cycle. During macronuclear development, histone H3 K9 methylation can target deletion of MIC-specific DNA in *Tetrahymena* (74, 130). Meiosis-specific Dicer- and Piwi-like proteins are required for the accumulation of a new class of small RNAs, scan RNAs (scnRNAs), a complex class of ~26–31-nt RNAs produced by meiotic micronuclei (80, 88, 91). These proteins are also required later for DNA elimination in the developing MAC. Together, these observations have prompted the suggestion that the programmed DNA-deletion patterns in *Tetrahymena* are programmed by scnRNA-directed methylation of histone H3 on lysine positions 9 and 27 on germline-specific sequences in the developing MAC (74, 89, 91). Histone methylation-dependent DNA elimination in ciliates may be analogous to heterochromatin-related transcriptional gene silencing in other eukaryotes (see the next section). This analogy is important because orthologous proteins may participate in both phenomena.

The so-called scan RNA model for programmed DNA deletion in *Tetrahymena* and *Paramecium* proposes that the whole micronuclear genome, or the sequence around IESs, is first transcribed bidirectionally during early conjugation, leading to the formation of long dsRNAs (89, 90) that are then processed to small RNAs by a Dicer-like enzyme. The precise localization of the nucleic acid information transfer and processing remains unknown. The name scnRNA implies that these small molecules may scan across DNA in the maternal MAC by exploiting base pairing to identify MIC-specific sequences. The scnRNAs would then accumulate in the parental MAC, where the scnRNAs that pair to a homologous macronuclear DNA sequence would be degraded. As a result, only scnRNAs homologous to MIC-specific sequences would be uncaptured and able to migrate to the newly developing MAC, where the scnRNAs could pair with matched regions of genomic sequence to target those DNA regions for elimination.

Recent studies in *Tetrahymena* and *Paramecium* have tested some of the predictions of the scan RNA model. First, the relocation of *Tetrahymena* scnRNAs from old to new macronuclei appears to occur in association with a Piwi-like protein, Twi1p, as supported by the finding that small RNAs coimmunoprecipitate with Twi1p both at stages of conjugation when most of the Twi1p is in the old MAC and at stages where most of the protein is in the new MAC (89). Second, the predicted enrichment of IESs in the scnRNA population during macronuclear development has been indirectly inferred through an observed increase in the ratio of small RNAs that hybridize to micronuclear DNA to those that hybridize to macronuclear DNA during the time course of conjugation (89). At later stages, this ratio decreases, which is consistent with the continuous production of small RNAs in these cells, except that the later-derived small RNAs do not enter the selection pathway to generate scnRNAs (89). In addition, microinjection experiments in *Paramecium* provided the first direct evidence that 25-nt small RNAs can promote IES excision (70).

Although the scan RNA model may explain how the maternal MAC can epigenetically guide DNA elimination in the new MAC, it cannot account for two features of DNA rearrangement in *Tetrahymena*. First, some mutations in the IES flanking sequences inhibit IES elimination (36). This feature suggests that in addition to RNA-mediated recognition of the IES sequence, the enzymatic machinery required for excision and rejoining of IES regions could have some sequence specificity, which may also be the case in *Chilodonella* (137). Another possibility is that the flanking sequences of IESs may be required for production of MIC-specific, nongenic transcripts. Second, transgenes present only in the MIC can be inherited by the new MAC in knockout heterokaryon strains (41).

Current experimental data implicate a possible combination of the scan RNA model with a model that involves scanning across maternal transcripts instead of the DNA itself. In other

words, the scnRNAs may scan against an RNA copy of the maternal MAC genome (Figure 5). The elusive mechanism by which ciliates recognize germline sequences for elimination may thus involve a natural genomic subtraction, which is computed *in vivo* by RNA–RNA base pairing. This possibility raises questions of specificity because RNA can tolerate a range of noncanonical base pairs. Most investigators of *Tetrahymena* and *Paramecium* have turned to a model that proposes scnRNA selection through interactions with long maternal RNAs (5, 70). The interaction between these two types of RNAs during development is key to our understanding of the large-scale process of genome reconstruction in ciliates.

A Role of Chromatin Structure During DNA Elimination

A difficult question concerning all types of DNA elimination during macronuclear development is how the genomic regions identified by the proposed RNA-mediated mechanisms are marked for elimination. Many observations point to a role of chromatin structure in this phenomenon. Some sequences that are eliminated during *Euplotes* macronuclear development adopt a unique chromatin structure (48). In spirotrichous ciliates (i.e., *Euplotes*, *Oxytricha*, and *Stylonychia*), macronuclear development begins with multiple rounds of DNA replication that result in the formation of polytene chromosomes. During the period of DNA elimination, the polytene chromosomes become compartmentalized, and DNA segments are destroyed throughout the chromosomes (59). Differences between eliminated and retained chromatin have been reported in *Stylonychia* (86, 87). During macronuclear development, distinct small (12-nm) and large (30-nm) chromatin fibers can be detected in the polytene chromosomes. The 30-nm fibers can form circular structures that separate from the chromosomes and subsequently degrade. The 12-nm chromatin fibers remain in the nucleus and eventually correspond in length to the typical macronuclear DNA chromosomes. These observations suggest that some of the DNA regions to be eliminated acquire a specific, highly compact chromatin structure that accompanies their excision in circular forms. Jahn et al. (49, 50) suggested that these circular forms in *Oxytricha* and *Euplotes* may derive from very large genomic regions located outside physical clusters of MDSs in the MIC.

More recent studies of *Tetrahymena* have provided additional evidence for the association of heterochromatin with DNA eliminated during macronuclear development. Studies of Pdd (programmed DNA degradation) proteins, which are located in the developing macronuclei and form large heterochromatic structures that appear during DNA elimination (23, 77, 78, 97, 98, 124), have shown by hybridization that these heterochromatic regions contain IESs. Moreover, Pdd proteins contain chromodomains, which are conserved in heterochromatin-associated proteins over a wide range of organisms (60). Notably, chromodomains do not directly bind DNA but rather interact with RNA (2). This observation further suggests a role for RNA in the process of heterochromatin formation and DNA elimination in *Tetrahymena*.

In general, studies of various ciliate species suggest that DNA elimination is preceded by a major reorganization of chromatin structure in the developing MAC. It seems clear that heterochromatin structure must be formed prior to DNA elimination and that the regions that are captured in the MAC probably remain in a much less compact form of chromatin. Extensive studies of histone modification in the developing MAC of *Tetrahymena* have revealed that a direct role of the Pdd1 protein is methylation of lysine 9 on histone H3 in the new MAC (130). Because chromatin studies in many other eukaryotes have revealed that methylated lysine position 9 on histone H3 is an epigenetic mark associated with silenced chromatin (8, 51, 62, 73, 95, 99), DNA elimination in the ciliate MAC may be the ultimate form of chromatin silencing, in which heterochromatin formation leads to complete elimination of the compacted DNA from the somatic nucleus.

EPIGENETIC PROGRAMMING OF DNA UNSCRAMBLING

The remarkably complex level of DNA rearrangements in ciliate species such as *Oxytricha* and *Stylonychia*, which eliminate nearly all of their germline DNA and possess scrambled genes, requires significantly more explanation and more DNA-processing steps than do *Paramecium* or *Tetrahymena*. Genome rearrangement in most stichotrich species involves permutations and inversions of DNA segments, in addition to simple deletions. Following the excision of all germline-specific DNA (which includes IESs, intergenic DNA, and transposable elements), the remaining MDSs must precisely assemble in the correct order and orientation (110). The whole process compresses the genome of *O. trifallax* from the original 1-Gb MIC to only ~50 Mb in the haploid content of the MAC. In addition to DNA unscrambling, massive chromosome fragmentation and amplification results in gene-sized nanochromosomes (44, 109) that are independently amplified to thousands of copies per MAC (42).

Some details of the mechanism of unscrambling are only now coming to light. This process, like other types of IES excision, also appears to involve recombination between short direct repeats, or pointers, and the repeats are typically longer between scrambled versus non-scrambled junctions (111). The sequence of the repeats varies between pairs of MDS ends, with no detectable consensus, although there may be enrichment for some motifs (66). The unscrambling machinery must therefore be able to recognize pairs of identical but distant MDS borders to join them together in the correct order. Prescott & Greslin (113) initially proposed an intramolecular unscrambling model based on homologous recombination between pointer repeats. In this model, both segment unscrambling and IES excision occur simultaneously through micronuclear DNA folding, permitting recombination to take place between two identical pointer repeats, one copy of which is retained in the macronuclear DNA molecule. This process would allow joining of the MDSs in the correct order and orientation. Current work with the scan RNA model, however, in *Tetrahymena* and *Paramecium*, has raised interest in possible roles of RNA in programming rearrangements in other ciliate species. However, although the scan RNA model can explain the programming of simple DNA deletions, its explanatory power falls short when it comes to genome unscrambling in *Oxytricha* or *Stylonychia*. The scnRNA process would merely highlight germline-specific genomic regions destined for deletion; it could not provide sufficient information to reorder DNA segments unambiguously, in part because of the very short length of scnRNA molecules, but also because some IESs in *Oxytricha* are even shorter than scnRNAs.

The Template RNA Model

The ability of hundreds of thousands of MDSs to rearrange into the correct linear order and orientation from generation to generation implies the existence of a controlled and precise rearrangement pathway, distinct from the scnRNA mechanism. Prescott et al. (112) and later Angeleska et al. (3) proposed a model in which a full-length maternal RNA or DNA template can orchestrate macronuclear DNA rearrangements. In this model, the long maternal template provides sufficient information to program deletions and to link contiguous fragments in the correct order. Macronuclear templates may provide a scaffold to organize the layout of segment order and DNA deletion through the use of strand displacement and branch migration to align pointer pairs for recombination (3).

Without access to a template, we conjectured that incorrectly spliced sequences (the results of promiscuous recombination at alternative pointers) would dominate, and we confirmed this hypothesis by use of RNAi against the putative templates (103). Mollenbeck et al. (93) also showed that a certain level of promiscuous recombination between short identical repeats can be a driving force in the production of errors during early development;

however, the multitude of such errors persisted into mature cells when their DNA processing was stalled in the RNAi experiments (103).

Using reverse-transcriptase polymerase chain reaction, our 2008 study (103) also included detection of both sense and antisense template transcripts in a brief window of time during *Oxytricha* development. Injecting mating cells with synthetic, alternative templates containing a novel MDS order provided the strongest test of the template model by permitting us to ask whether these molecules could reprogram the order of rearranged segments. The sequence of each injected molecule corresponded to a complete telomere-to-telomere copy of an altered nanochromosome with a new MDS order, and the progeny of injected cells did inherit the alternative MDS pattern in their macronuclear genome. Moreover, the effect was reproducible regardless of whether we injected sense, antisense, or both RNA strands, or even DNA (which we assume was transcribed in vivo). This study provided the first molecular evidence that sense and antisense RNA copies of complete maternal macronuclear chromosomes are present and capable of programming DNA rearrangement and assembly of new macronuclear chromosomes in *O. trifallax* (Figure 6).

The 2008 study also demonstrated the effect on more than one gene, and Figure 7 provides an additional example that extends these results to RNA-programmed DNA inversions, which also occur in gene unscrambling. We conclude that RNA templates inherited from the maternal MAC (or from a foreign source in the environment) can guide the assembly of new macronuclear chromosomes and, thus, permit epigenetic inheritance of alternatively rearranged chromosomes or DNA inversions. Additionally, a subset of the mutations that were originally introduced into the alternative templates to distinguish the microinjected templates from the processed endogenous molecules did transfer to the latter, both in our previous study (103) and in the experiment depicted in Figure 7. These results implicate the involvement of RNA template-guided DNA repair. Taken together, these experiments reveal a new class of noncoding RNAs (template RNA) and demonstrate the ability of RNA templates to guide genome rearrangements and to transfer heritable information via non-Mendelian, epigenetic means, thereby extending the functional repertoire of RNA.

Evolutionary Considerations

An important outcome of these studies was the observation that reprogrammed DNA rearrangements can epigenetically transfer to subsequent sexual generations, at least as far as the F₃ generation (103). This transgenerational inheritance of DNA rearrangements demonstrates that these eukaryotic cells can pass information across generations via RNA. It also validates an earlier prediction (108) that a “backup copy of [the] grandparents’ genetic information encoded in RNA” can transmit instructions to the progeny, even though the original context was inheritance of a proposed RNA cache that permitted genotype reversion in *Arabidopsis* (75), a phenomenon later attributed to out-crossing (83).

The evolutionary consequences of a viable mechanism in ciliates to transmit acquired characters may create an additional store of heritable variation and contribute to the cosmopolitan success of this diverse lineage of microbial eukaryotes. Thus, changes that arise in the maternal somatic genome, whether at the level of point mutations or new DNA-rearrangement patterns, have an opportunity to be passed on to the next generation, thereby bypassing the usual mode of inheritance via germline DNA. Furthermore, the occasional transfer of point mutations from RNA templates to the next sexual generation provides a mechanism for the stable inheritance of acquired, spontaneous somatic substitutions without alteration of the germline genome. Both of these RNA-mediated events—programmed DNA rearrangements and transfer of point substitutions—set up a potential duality between the germline genome and the somatic, transcribed epigenome, a duality that deep sequencing of

both genomes from a single cell may detect in the form of alleles present in one genome and not in the other.

We propose that RNA-driven DNA inversion (Figure 7) may be a novel mechanism for the origin of some DNA inversions in genomes. When an inversion first occurs at the RNA level through a rogue RNA-splicing event, the RNA containing the inversion can serve as a template to guide DNA inversions. If such a cascade of events were to occur in the germline, it would produce a heritable inversion polymorphism. In somatic cells, such events could contribute to the phenomenon of recurrent DNA inversions in the human genome (30). Deep RNA sequencing could reveal the portion of somatic DNA inversions or other DNA rearrangements that could be driven by RNA-mediated DNA rearrangement (3). Rowley & Blumenthal (119) proposed this as a possible mechanism to explain the association of a DNA translocation in human endometrial tumor cells with the regulated presence of a chimeric *trans*-spliced mRNA (*JAZF1-JJAZ1* RNA) in normal endometrial cells that lack the translocation (15, 72).

Several groups have studied the origin and evolution of scrambled genes in different phyla (24, 46, 64, 125, 127), and we have written about it at greater length elsewhere (19, 64, 65). Some of the mysteries that remain include the evolutionary events that trigger scrambling in the germline and whether they occur in a gradual or punctuated fashion. Ectopic recombination between IESs or transposons is one possibility in a stepwise model, as is recombination between short, cryptic repeats present in both coding regions and IESs (19). Any series of such events could serve to jumble segment orientation and order and would permit expansion of a scrambled germline architecture, once established. Another possibility is that a cataclysmic event, such as chromothripsis (129), could suddenly scramble one or more loci in the MIC or MAC, as in our experimental manipulations in the laboratory (103). The subsequent RNA templates transcribed from a rearranged macronuclear DNA molecule would propagate the altered DNA rearrangement pattern in sexual offspring, as we demonstrated via epigenetic reprogramming of a gene-rearrangement pathway over two generations (103). Conversely, if a chromothripsis-like event were to scramble several loci in the MIC, the cell would still produce RNA templates from the MAC to restore working genes in its sexual offspring. Thus, RNA-guided DNA recombination can preserve genome integrity.

RNA recombination via *cis*- or *trans*-splicing of the template RNAs themselves could also introduce new DNA-rearrangement pathways in the offspring. Such events would occasionally produce a new, scrambled interpretation of the relevant germline loci, and this information could transfer across generations via the template model (Figure 6), allowing epigenetic creation of a new scrambled gene (or genes). One would expect such novel rearrangements in the MAC to persist only if they confer a selective advantage to the offspring. Fortunately, genomic or proteomic studies will allow us to test whether any creative DNA arrangements, similar to exon shuffling, have contributed to the gene richness and diversity of ciliate proteomes. This hypothesis predicts that the germline architectures of novel genes (those that lack orthologs in nonciliate or nonstichotrich species) are more likely to be scrambled than those that possess orthologs in several species.

MATERNAL RNA CONTROL OF DNA AMPLIFICATION

Very recent discoveries revealed that the role of maternal RNA templates in nuclear development in *Oxytricha* and *Stylonychia* goes beyond DNA unscrambling and IES excision. In these two ciliates, maternal RNA seems to regulate chromosome amplification as well (43, 100). As mentioned above, the process of micronuclear chromosome fragmentation in spirotrichs produces thousands of gene-sized macronuclear

nanochromosomes, which then amplify independently to a copy number ranging from hundreds to tens of thousands. As a result, a single ciliate MAC may contain millions of short DNA chromosomes (7, 109). In both *Oxytricha* and *Stylonychia*, microinjection of single-stranded RNA molecules that correspond to various macronuclear chromosomes during conjugation results in a higher chromosome copy number in the sexual progeny. Conversely, the use of RNAi to reduce the concentration of maternal RNA during conjugation leads to a reduction in copy number of the corresponding chromosome in the next generation. As with the reprogrammed DNA rearrangements, the effect can be transgenerational (43, 100) and is specific for the homologous chromosome, with no observed effect on the copy number of other DNA molecules.

The results strongly suggest maternal inheritance of somatic chromosome copy number in spirotrichous ciliates. It is possible that the same maternal RNA templates that guide DNA rearrangements also have a quantitative influence on chromosome replication during development. An epigenetic model for RNA-mediated DNA copy number regulation proposes that the concentration of RNA templates produced in the parental nucleus is proportional to their chromosome copy number at the time of cell mating. After their transport to the developing nucleus, the template RNAs can provide the set of epigenetic instructions to guide genome rearrangements. As a result, the level of correctly processed chromosomes may simply be proportional to the concentration of available RNA templates. This hypothesis makes the testable prediction that a quantitative correlation exists among the relative copy numbers of maternal DNA, maternal RNA, and progeny DNA. It may also explain the observation that chromosome copy number is established very early during macronuclear development in spirotrichs (109). However, the imprecise nature of amitotic nuclear division does lead to fluctuations of individual nanochromosome copy numbers during clonal growth. The high ploidy serves as a temporary buffer, but after dozens of cell divisions, the chromosome copy numbers may vary significantly from the initial, inherited ones. This possibility is consistent with the observation that *O. trifallax* cells are unable to produce viable progeny after 80 divisions (1), which could be partly due to changes in maternal DNA content. The presence of single-gene chromosomes in some species creates a unique opportunity to regulate gene expression at the level of individual chromosome copy number, without interfering with others. Epigenetic control of gene copy number introduces an enormous plasticity into genomes and may help the cell adapt to environmental changes, but this opportunity also comes with trade-offs because it may be coupled to senescence and requires its own regulation.

CONCLUDING COMMENTS

Recent discoveries of RNA-mediated epigenetic phenomena in ciliates show that maternal cells regulate nearly every step in their development. By itself, the archival germline genome, although much richer than the soma in terms of its coding information, is woefully inadequate to program the cell's development. Some of the elegant solutions that species such as *O. trifallax* have evolved include stripping its archival genome of nearly all noncoding DNA to streamline the tasks of vegetative growth; however, these organisms require considerable help from previous generations to accomplish this heroic task. We can look forward to finding elements of RNA-mediated inheritance in other eukaryotes as well, perhaps even within ourselves, a discovery that will have consequences for understanding the events leading to chromosomal rearrangements and inversions in somatic or germline cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

An example of the ciliate sexual cycle. (a) A vegetative *Paramecium* cell. (b) Meiosis of two micronuclei. The macronucleus begins its fragmentation. (c) Seven out of eight haploid meiotic products degenerate; one (shown at *top*) divides mitotically, producing two identical gametic nuclei. During conjugation, one of the gametic nuclei is exchanged between partner cells. In the case of autogamy (self-fertilization), the two identical gametic nuclei fuse together. (d) A cell that contains the zygotic nucleus. (e) The zygotic nucleus undergoes two subsequent mitotic divisions. (f) Two of the products differentiate into new macronuclei. (g) Two karyonidal cells are present; each contains one new macronucleus, two micronuclei, and fragments of the old macronucleus.

Figure 2.

Types of genome rearrangements during macronuclear development in ciliates. The micronuclear genome undergoes amplification to varying levels between and sometimes within different species, and three types of DNA elimination can occur (not necessarily in the order shown). One is imprecise elimination of germline-specific repeats, including all transposons and minisatellites. This step is often associated with chromosome fragmentation and healing of chromosome ends by de novo telomere addition (*red*). The second type of rearrangement is the precise excision of internal eliminated sequences (IESs, *yellow*), which occurs in both *Paramecium* and *Oxytricha*. Stichotrichs, such as *Oxytricha*, also have a third layer of descrambling segment order and orientation. Precise DNA elimination and/or unscrambling restores the coding regions to construct functional open reading frames (*thick black arrow*). Abbreviations: MIC, micronucleus; MAC, macronucleus.

Figure 3.

Mendelian and non-Mendelian inheritance in *Paramecium*. (a) Mendelian pattern of allelic segregation in a cross between A/A and a/a homozygotes. Conjugation produces two genetically identical heterozygotes, A/a . Autogamy of the heterozygous clones results in entirely homozygous clones with a 50% chance of receiving each of the parental genotypes. (b) Non-Mendelian maternal inheritance of mating types in *P. tetraurelia*. Each mating type (O or E) follows a cytoplasmic pattern of inheritance that is determined by the macronuclei. Mating type is programmed at every sexual reproduction by the maternal macronucleus via the maternal cytoplasm. Figure based on experiments in Reference 85.

Figure 4.

Epigenetic inhibition of internal eliminated sequence (IES) excision. (a) In wild-type cells, all IESs and transposons (*orange*) are removed during development of the new macronucleus (MAC). Tiny red lines indicate telomeres at MAC chromosome ends. During sexual reproduction, the parental MAC is lost, and the new MAC develops from the parental micronucleus (MIC). (b) Microinjection of the MAC with an IES sequence (*short orange bar* matching the genomic IES sequence on the right) specifically blocks excision of this IES in the MAC of sexual progeny (26).

Figure 5.

The scan RNA model for programmed genome rearrangements. (a) Endogenous short RNAs (scnRNAs, *blue and orange dashed lines*) are produced in the meiotic micronucleus (MIC). (b) These scnRNAs are exported to the maternal macronucleus (MAC), where they may scan the genome via interactions with long maternal transcripts (*blue wavy lines*). The scnRNAs at this point correspond to the entire germline genome, including transposons and internal eliminated sequences (IESs, both in *orange*). (c) scnRNAs (*orange dashed lines*) that cannot pair with homologous sequences are then selectively transported to the developing (zygotic) MAC, where they target histone methylation on homologous DNA sequences. (d) The process in panel *c* results in the specific elimination of micronuclear sequences that are absent from the maternal MAC. Tiny red lines indicate telomeres.

Figure 6.

A template model for RNA-guided genome rearrangements in *Oxytricha*. (a) Bidirectional maternal template transcripts (green wavy line) are produced from short macronuclear chromosomes and then transported to the developing macronucleus (MAC). (b) Here, the RNA transcripts act as scaffolds to guide rearrangements (deletion, permutation, and inversion) of corresponding micronuclear DNA sequences. (c) This process leads to the complete elimination of micronucleus (MIC)-specific DNA (orange boxes). (d) De novo telomere addition (red boxes) and amplification complete the formation of new macronuclear chromosomes.

Figure 7.

RNA-mediated epigenetic reprogramming of a DNA inversion in *Oxytricha*. This example illustrates a new scrambled pattern in the nonscrambled gene encoding telomere end-binding protein subunit β (*TEBP β*) that arises from the introduction of a synthetic RNA template containing an inversion. (a) To create the artificial template, macronucleus-destined sequence (MDS) junctions flanking target segment 4 were slightly adjusted to program two new recombination breakpoints, accommodating short existing repeats as new pointers (TGGT between the 3' end of segment 3 and the 3' end of segment 4, and CTTC to fuse the 5' end of segment 4 to the 5' end of segment 5; underlined sequences are embedded in the wild-type ACTTC pointer between MDS 4 and 5; the TGGT breakpoint begins 5 bp upstream of the wild-type ACTC pointer between MDS 3 and 4). The resulting alternative, 1.8-kb template containing inverted segment 4 (*inv4*) is 27 nt shorter than wild-type *TEBP β* . Two independently microinjected constructs, *inv4*⁺ and *inv4*²⁺, contain different numbers of substitutions within 36 nt of the inverted junctions to distinguish injected molecules from endogenous, rearranged progeny DNA and to query substitution transfer. (b) Genomic DNA extracted from the F₁ progeny of cells injected with either sense (s) or antisense (as) template transcripts were amplified by polymerase chain reaction (as in 103) through the use of the primers indicated by arrows in panel a. The 231-bp product contains the inversion (*inv*) relative to the 258-bp wild-type (WT) product in noninjected control cells (ctrl). M indicates the 1-kb plus DNA ladder (Invitrogen, Carlsbad, California). This example illustrates that efficient epigenetic reprogramming of the DNA-rearrangement pathway occurs in this gene, although the WT version is also present. DNA sequencing confirmed that the 231-bp products contain endogenous, rearranged molecules with the programmed DNA inversion. Sequences are provided in the Supplemental Appendix (follow the Supplemental Material link from the Annual Reviews home page at <http://www.annualreviews.org>).