RNA Polymerase II Localizes in Tetrahymena thermophila Meiotic Micronuclei When Micronuclear Transcription Associated with Genome Rearrangement Occurs

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Tetrahymena thermophila shows nuclear dimorphism (reviewed in references 8 and 24). Each cell contains a germ line micronucleus and a somatic macronucleus. Although both nuclei are derived from the micronuclei during conjugation, the micronucleus and a somatic macronucleus. Although both nuclei are derived from the micronuclei during conjugation, the macronucleus lacks ~15% of the micronuclear genome due to two types of sequence elimination that occur during macronuclear development. One is deletion of ~6,000 internal eliminated sequences (IES), accompanied by ligation of flanking macronucleus-destined sequences. IES in Tetrahymena vary from 0.5 to >20 kb and account for most of the sequences missing in the macronucleus. Excision of IES at a specific site can occur reproducibly or with a limited number of alternative boundaries and is epigenetically regulated by the old macronucleus (2). The other type of elimination involves chromosome breakage at specific 15-bp chromosome breakage sequences, followed by small (<50-bp) deletions of breakage-eliminated sequences (BES) and the addition of telomeres to produce 200 to 300 macronuclear chromosome sequences from the 5 chromosomes in the micronuclear (haploid) genome.

The micronucleus is believed to be transcriptionally silent in vegetatively growing cells (5). However, nongenic micronuclear transcription has been detected early in conjugation (11, 23), when premeiotic micronuclei adopt an elongate crescent shape that probably is related to “bouquet” or “horsetail” stage in other eukaryotes (17). RNA hybridizing to a micronuclear-specific sequence was detected in starved and mating cells (22), and long, heterogeneous RNAs homologous to both strands of IES have been observed during conjugation (3). Other features associated with transcription have also been localized to crescent micronuclei. At meiotic prophase, TATA-binding protein also first localizes in micronuclei (21). Thus, a general transcription system probably starts localizing to micronuclei at this stage. In addition, chromatin remodeling occurs in micronuclei at this stage of conjugation. Histone variant H2A.Z (formerly called hv1) and acetylated histones, hallmarks of actively transcribed chromatin, start localizing in the micronucleus during meiotic prophase (20).

Recent studies implicate an RNA interference (RNAi)-related mechanism in genome rearrangement in Tetrahymena (reviewed in reference 14). Twi1p, a member of the PPD protein family involved in diverse RNAi processes, was shown to be required for genome rearrangement (13). Twi1p interacts with and is required for the accumulation of conjugation-specific small RNAs (13, 15). Also, injection of double-stranded RNA (dsRNA) can induce ectopic DNA elimination (25). A scan RNA (sncRNA) model has been proposed that explains how IES can be eliminated in the absence of consensus sequences by an RNAi-related mechanism and accounts for the observed epigenetic regulation (13, 14). In this model the micronuclear genome is transcribed bidirectionally in early conjugation to form dsRNAs that are processed to small sncRNAs by an RNAi-like machinery. The sncRNAs then accumulate in the (parental) macronucleus where those having homology to macronuclear DNA sequences are degraded. As a result, only sncRNAs homologous to micronucleus-specific (IES or BES) sequences remain in the old macronucleus. Finally, according to this model, these IES- or BES-homologous sncRNAs move to the developing new macronucleus. There, sequences homologous to sncRNAs are identified as IES or BES and targeted for elimination. In this model, transcripts made in the early meiotic micronucleus play central roles in the genome rearrangements that occur in the newly developed macronucleus. cnjC was identified as a gene expressed during conjugation...
but not in vegetative cells (12). Because cnjC mRNA appeared to be specifically expressed when crescent transcription occurred and CnjCp was similar to some subunits of RNA polymerases (RNAPs) (10), we set out to determine whether this gene was specifically involved in production of the transcripts in crescent micronuclei that gave rise to scRNA's. However, we discovered that cnjC expression is not conjugation specific. Rather, it is the only gene in the Tetrahymena genome that encodes the conserved, third largest subunit of RNAP II, and we have, therefore, renamed this gene RPB3. Rpb3p is expressed in vegetative cells at low levels, localizes to micronuclei at all stages of the life cycle, and is required for vegetative growth. Nonetheless, Rpb3p localizes in the micronucleus only during meiotic prophase, where its high concentration and similar localization to ongoing RNA synthesis (11, 23) argue that RNAP II is involved in crescent transcription.

**RESULTS**

RPB3 is a unique gene that encodes the third largest subunit of RNAP II. Previous studies suggested that RPB3 (cnjC) encoded a protein related to Rpb3p, the third largest subunit of Saccharomyces cerevisiae RNAP II, and to prokaryotic RNAP (10). We identified an RPC5 homologue (the shared, fifth largest subunit of RNAPs I and III, also known as RPC40 in yeasts and RPC40 in humans) in the unpublished Tetrahymena genome database as well as in an expressed sequence tag database. Phylogenetic analysis indicated that Rpb3p and Rpe5p were related to Rpb3p and Rpe5p homologues, respectively, in other eukaryotes (Fig. 1A).

We failed to identify other Rpb3p homologues in the Tetrahymena genome database, which should be nearly complete and has not failed to contain any previously known gene sequence. Thus, RPB3 is likely to be the only Tetrahymena gene encoding the third largest subunit of RNAP II.

**RPB3 expression is not restricted to conjugation.** RPB3 (cnjC) was described as a gene specifically expressed during early conjugation (12). However, this result is puzzling in light of our finding that RPB3 encodes the only third largest subunit of RNAP II in Tetrahymena. Therefore, we reexamined...
mRNA expression (Fig. 1B). RPB3 mRNA was expressed strongly at early conjugation stages as previously reported. RPB3 expression was similar to that of TWI1 (Fig. 1B), which is involved in small RNA accumulation and genome rearrangement (13) and is identical to a cDNA (cnjC) (GenBank accession number AY129082) identified in the same screen that identified cnjC (12). RPB3 mRNA also was detected in growing cells and at very low levels in stationary phase and starved cells (Fig. 1B). This is not likely to be due to cross-hybridization with other genes because, as described below, Rpb3p was also observed in vegetatively growing cells (see Fig. 3C and 4).

Thus, RPB3 mRNA expression is not restricted to conjugating cells but also occurs in vegetative cells where its low level of expression probably explains its failure to be detected in the original studies.

RPB1, which encodes the largest subunit of RNAP II (19), was also expressed in log-phase and stationary-phase cells and, like RPB3, was expressed at an increased level during conjugation (Fig. 1B). Thus, the expression patterns of RPB3 and RPB1 are similar, except that RPB3 is not up-regulated in stationary-phase cells, and likely reflect the pattern of expression of all RNAP II subunits. In contrast, RPC5 expression was detected in log-phase growing vegetative cells and weakly in early stages of conjugation. Thus, de novo synthesis of RNAPs I and III should be low in conjugating cells.

RPB3 is essential for vegetative growth. If RPB3 is the only gene encoding a subunit of RNAP II in *Tetrahymena*, it is expected to be essential for vegetative growth. To address this question, the RPB3 gene was disrupted. The knockout construct (Fig. 2A) was introduced into conjugating *Tetrahymena* strains to give germ line heterozygous RPB3 knockout heterokaryons strains with micronuclei in which one of the two copies of the RPB3 gene was replaced with the knockout construct by homologous recombination (Fig. 2A). Two germ line heterozygous heterokaryons were mated, and the genotypes of the paromomycin-resistant progeny were analyzed by PCR (Fig. 2B). If RPB3 is not essential, one-third of the paromomycin-resistant progeny should be homozygous RPB3 knockout cell (Fig. 2B) (one-fourth of the progeny should be wild type, but they are paromomycin sensitive). However, all 24 progeny analyzed were heterozygous RPB3 knockout cells (Fig. 2B), suggesting that RPB3 is essential for vegetative growth.

To confirm this result, germ line homozygous RPB3 knockout heterokaryons were constructed (see Materials and Methods) with micronuclei in which both of the RPB3 genes were replaced with the knockout construct but with the wild-type RPB3 gene in the macronuclei, and their progeny were analyzed. Because both copies of the RPB3 loci in the micronucleus are disrupted in the germ line homozygous RPB3 knockout heterokaryons, all progeny derived from this mating are homozygous homokaryons whose RPB3 genes are disrupted in both macro- and micronucleus genomes. As expected if RPB3 is an essential gene, no progeny were obtained from $\sim 2 \times 10^6$ exconjugants from a mating of two germ line homozygous RPB3 knockout heterokaryons, although we could easily obtain progeny from wild-type cells under the same conditions. Individual pairs of a mating between two germ line homozygous RPB3 knockout heterokaryons were isolated into drops of culture medium, and the number of progeny cells produced was determined. Most of the exconjugants divided once but eventually died (Fig. 2C). Because maternally expressed Rpb3p can be detected in the zygotic macronucleus (see Fig. 4), the cell division(s) observed in the progeny of the germ line homozygous RPB3 knockout heterokaryons probably reflects utilization of maternal Rpb3p. These results confirm that RPB3 is essential for vegetative growth, consistent with the view that RPB3 is the only gene that encodes the third largest subunit of RNAP II in *Tetrahymena*.

Expression and localization of Rpb3p. To observe the localization of Rpb3p, we replaced the endogenous RPB3 gene with...
a transgene that encodes Rpb3p tagged with HA at the C terminus (RPB3-HA) (Fig. 3A). All endogenous RPB3 genes in the macronucleus could be replaced by RPB3-HA (Fig. 3B), demonstrating that RPB3-HA is functional.

By Western blotting (Fig. 3C), a protein whose molecular mass (36 kDa) was similar to that predicted for Rpb3p-HA (37.1 kDa) was detected by using anti-HA antibody. Rpb3p-HA was observed at all stages of the life cycle exam-
ined, consistent with the essential function of RNAP II in transcription. The amount of Rpb3p did not appear to increase greatly during conjugation and clearly did not increase to the same extent as the mRNA, possibly because the macronuclear Rpb3p (see below) synthesized by vegetative cells is stable.

Rpb3p-HA was detected by immunofluorescence staining in macronuclei in all stages of the life cycle examined (Fig. 4). Staining of micronuclei was not detected in log-phase growing cells or in starved cells (Fig. 4A and B). Strikingly, in the early stages of conjugation, Rpb3p-HA rapidly localized in the micronucleus (Fig. 4). It was first observed in early conjugation stages (stage I, leptotene) (Fig. 4C) just before elongation and continued to be localized in micronuclei (Fig. 4C to G) until they were fully elongated (stage IV, pachytene) (Fig. 4G). Because an RPB3-HA strain and a wild-type strain were mated in this experiment, staining was first observed only in one cell of the pair (Fig. 4C). However, Rpb3p-HA appeared rapidly in the micronucleus (Fig. 4C and D) and gradually appeared in the old macronucleus (Fig. 4E and F) of the untagged cell. In the macronucleus, a core spot lacking Rpb3p-HA staining was observed. This probably corresponds to the chromatin-dense region described previously (23). At stage III (zygotene; micronucleus elongated to almost the same length as cell length), Rpb3p-HA staining began to disappear from the central part of the crescent (Fig. 4E) until, at stage IV, only a small portion of the crescent stained at one end (Fig. 4F and G). Staining became undetectable when chromosome condensation occurred prior to meiosis I (Fig. 4H). These localizations are quite similar to the sites where crescent transcription has been observed (11, 23), indicating that RNAP II is involved in crescent transcription.

Rpb3p-HA localized in parental macronuclei throughout conjugation (Fig. 4C to J) until the new macronuclei developed, at which time it localized to newly developed macronuclei, while staining of old macronuclei rapidly disappeared (Fig. 4K).

RNAPs I and III are not detected in micronuclei. The data above indicate that RNAP II is localized in the early meiotic micronucleus and could be involved in crescent transcription. To determine whether this is specific for RNAP II or general for RNAPs, we examined the localization of HA-tagged Rpc5p, the fifth largest subunit shared in RNAPs I and III (Fig. 5A). RPC5-HA could completely replace the endogenous RPC5 loci in the macronucleus (Fig. 5B). We have not tested whether RPC5 is an essential gene in Tetrahymena. However, because RPC5 is the only gene encoding the eukaryotic Rpo40 homologue in the Tetrahymena genome and Rpo40 is indispensable for cell viability of budding yeast (9), it is likely that RPC5 is essential for cell viability in Tetrahymena. Thus, Rpc5p-HA should reflect the localization of endogenous Rpc5p.

As expected, Rpc5p-HA was localized in macronuclei in vegetative cells (Fig. 6). When an RPC5-HA strain was crossed with a wild-type strain, Rpc5p-HA remained localized in the macronucleus; Mi, micronucleus; An, anlagen or new macronucleus. Arrows indicate the core region in micronuclei (see text). See references 4 and 23 for the stages of conjugation and of meiotic prophase, respectively.
macronucleus in only one of the paired cells and was not transferred to the other cell (Fig. 6). Rpc5p-HA was not detectable in micronuclei in any stage of the life cycle examined (Fig. 6). Thus, RNAP II is probably the only known RNAP that can be involved in micronuclear transcription during conjugation. While we cannot rule out a possibility that RNAPs I and III are localized in the micronucleus at low levels that cannot be detected by our method, the relative intensities of Rpb3p-HA in macro- and micronuclei (micronuclear staining > macro-nuclear staining) compared to those of Rpc5p-HA (easily detectable in macronuclei but not detectable at all in micronuclei) argue that polymerase II is likely to be responsible for the RNA synthesis observed autoradiographically in crescent micronuclei, which occurs at levels comparable to that for macronuclear transcription (11, 23).

**DISCUSSION**

**RPB3 (cnjC)** is not conjugation specific and is required for vegetative growth. Although RPB3 (cnjC) was first identified as a gene expressed only during conjugation, our analysis revealed that this gene is expressed at a low level in vegetative stages (Fig. 1B). RPB3 is essential for vegetative growth (Fig. 2) and is probably the only gene encoding the third largest subunit of RNAP II. Thus, we conclude that RPB3 expression is not specific to conjugation. Rather, RPB3 is expressed throughout the life cycle as a general subunit of RNAP II.

**RNAP II is probably involved in crescent transcription.** Rpb3p-HA was localized in macronuclei in all stages of the life cycle examined (Fig. 3C and 4). This is also consistent with the view that RPB3 is a general RNAP II subunit. Strikingly, Rpb3p-HA appeared in micronuclei in meiotic prophase (Fig. 6). While it is possible that Rpb3p (but not all RNAP II subunits) is localized in the meiotic micronucleus and that micronuclear Rpb3p is part of a transcriptionally inactive RNAP II, this seems highly unlikely. The fact that the localization of Rpb3p-HA in micronuclei was similar to the sites where RNA synthesis was observed autoradiographically by using [3H]uridine (11, 23) argues that Rpb3p in the meiotic micronucleus is in active RNAP II. In contrast, Rpc5p-HA, the fifth largest subunit of RNAPs I and III, was not detected in the micronucleus (Fig. 6). These results suggest that RNAP II, but not RNAPs I and III, is involved in crescent transcription. Transcription of several micronucleus-specific sequences has been detected during conjugation (3). These transcripts are heterogeneous in size and probably are not polyadenylated (3), making them different from typical, macronuclear RNAP II
transcripts. Although it is reasonable to expect that RNAP II localized in the micronucleus must contain subunits essential for transcription, it is not known if micronuclear RNAP II has exactly the same subunit composition as RNAP II in the macronucleus. Given that micronucleus-specific transcripts of specific sequences are extremely heterogeneous (3), it seems likely that micronucleus-specific RNAP II subunits, posttranslational modifications, or associated transcription factors might be involved in micronuclear transcription.

We observed a core region where Rpb3p-HA staining was not detected in the micronuclei during early conjugation (Fig. 4). Does this mean that not all of the micronuclear genome is transcribed? This issue bears on the nature of the micronuclear transcripts and their role in the process of DNA rearrangement. If the answer is yes, micronuclear transcription is specific, and the nature of the transcribed and untranscribed sequences and their relationship to IES and BES require additional study. However, three other explanations are also possible. First, the core region, though detectable cytologically, may not correspond to specific chromosomal regions; rather, it could represent regions that have not yet (or have already) been transcribed during meiotic prophase. Second, because the core region is a chromatin-dense region, the unstained spot may be an artifact caused by poor accessibility of the antibody. Third, the core could be aggregates of specific chromosomal regions, such as micronuclear telomeres, which are not transcribed because telomeres are added de novo during macronuclear development. Unfortunately, resolution of the sites of RNA synthesis determined by using the incorporation of [3H]uridine and light microscopic autoradiography was not sufficient to determine whether the core spot was transcribed or not.

What enables Rpb3p localization in the micronucleus? Because the amount of RPB3 mRNA is greater in conjugating cells than in vegetative cells (Fig. 1B), one possible explanation for the accumulation of Rpb3p in micronuclei is that macronuclear sites become saturated and the excess accumulates in the micronuclei in the conjugating cells. However, two observations argue that Rpb3p is actively localized to the micronucleus. First, compared to the RPB3 mRNA level, expression of the protein, Rpb3p, was not increased significantly during conjugation (compare Fig. 1B and 3C). Second, when the RPB3-HA strain was mated with a wild-type (nontagged) strain, Rpb3p appeared in the micronucleus of the wild-type partner much faster than in the macronucleus (Fig. 4C to F). Thus, Rpb3p is probably actively transported into micronuclei or micronuclei have stronger affinity for Rpb3p than the macronuclei during meiotic prophase. The increase in RPB3 mRNA in early conjugation and its rapid appearance in micronuclei suggest that Rpb3p is translated during early conjugation. In contrast, levels of RPC5 mRNA remain low at this time, and in the absence of growth, it is likely that there is little or no translation of Rpc5p in early conjugation. Thus, it is possible that de novo synthesis of proteins is required for their localization in the micronucleus as well as for their transfer to the macronucleus of the mating partner cell. The seemingly contradictory observations that the up-regulation of RPB3 mRNA (Fig. 1B) occurs without significant increase of Rpb3p (Fig. 3C) during early conjugation may simply reflect the fact that the total amount of newly synthesized micronuclear Rpb3p is small relative to the amounts that preexist in the macronucleus.

Is micronuclear transcription required for the genome rearrangement? In the scnRNA hypothesis, we proposed that micronuclear transcripts were processed by an RNAi-related mechanism to produce small RNAs involved in genome rearrangement. Because RPB3 is required for vegetative growth and probably for conjugation, we could not test whether RPB3 is required for the micronuclear transcription that leads to genome rearrangement. To rigorously test this hypothesis will require a mutation that prevents RNAP II from localizing in the micronucleus without affecting the transcriptional activity of RNAP II in the macronucleus. Nonetheless, for several reasons we think it highly likely that RNAP II is responsible for the micronuclear transcription that leads to IES elimination. First, as described here, RNAP II, but not RNAP I or RNAP III, appears in micronuclei when crescent transcription starts. Second, a Dicer-like protein is also localized to the micronucleus at early meiotic stages (our unpublished results). Third, double-stranded transcripts, a required substrate for the formation of small RNAs by Dicer-like enzymes, have been detected (3). Fourth, small RNAs homologous to IES have been detected (13). Fifth, a PPD protein (Tw1p) homologous to proteins involved in RNAi-like systems in other organisms is required both for the accumulation of small RNAs and for IES and BES elimination (13). Finally, dsRNA injected into conjugating cells can lead to IES elimination (25). Thus, the evidence linking RNAP II transcription in crescent micronuclei to scnRNA-mediated DNA rearrangement in Tetrahymena, though circumstantial, is compelling (14).

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