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Genetics

Antisense ribosomes: rRNA as a vehicle for antisense RNAs

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ABSTRACT **Although rRNA has a conserved core structure, its size varies by more than 2000 bases between eubacteria and vertebrates, mostly due to the size variation of discrete variable regions. Previous studies have shown that insertion of foreign sequences into some of these variable regions has little effect on rRNA function. These properties make rRNA a potentially very advantageous vehicle to carry other RNA moieties with biological activity, such as "antisense RNAs." We have explored this possibility by inserting antisense RNAs targeted against one essential and two nonessential genes into a site within a variable region in the *Tetrahymena thermophila* large subunit rRNA gene. Expression of each of the three genes tested can be drastically reduced or eliminated in transformed *T. thermophila* lines containing these altered rRNAs. In addition, we found that only antisense rRNAs containing RNA sequences complementary to the 5′ untranslated region of the targeted mRNA were effective. Lines containing antisense rRNAs targeted against either of the nonessential genes grow well, indicating that the altered rRNAs fulfill their functions within the ribosome. Since functional rRNA is extremely abundant and stable and comes into direct contact with translated mRNAs, it may prove to be an unparalleled vehicle for enhancing the activity of functional RNAs that act on mRNAs.**

rRNA is a mosaic of evolutionarily conserved and variable regions (1). The large subunit rRNA can vary in size by as much as about 2000 bases among free-living organisms, almost entirely due to size variation of a handful of variable regions (2). The divergence of sequence and size seen in variable regions suggests that substantial changes in their sequences might be tolerated, even within a given organism. Indeed, insertion of foreign sequences into some variable regions has little or no effect on rRNA function in *Tetrahymena thermophila* (3, 4). These data suggest that functional rRNA could serve as a vehicle to carry other functional RNA moieties, such as antisense RNAs, ribozymes, or protein binding sites. Since rRNA is among the most (if not the most) abundant and stable RNAs in the cell, and since it comes into direct contact with all translated mRNAs, it might be a very effective vehicle to enhance the activity of an inserted RNA sequence, especially one that acts on mRNAs.

Antisense RNAs (or DNAs) that are complementary to all or part of a target mRNA can reduce specific gene expression *in vivo* (5, 6). However, extensive studies have suggested that this inhibitory effect can be variable and unpredictable. The reasons have not always been clear, but may include low copy number, instability, and/or inappropriate intracellular localization of antisense molecules (6, 7). An antisense RNA embedded within the rRNA could avoid these problems and might offer robust and consistent antisense effects. In this study, we determine whether antisense RNAs embedded within the *T. thermophila* large subunit rRNA can efficiently inhibit target gene expression *in vivo*.

T. thermophila is a particularly suitable organism for the study of the rRNA genes (rDNA). It is a single-celled eukaryote containing two nuclei: a diploid micronucleus that is transcriptionally silent and a polyploid macronucleus that is actively transcribed. During conjugation, the micronucleus undergoes meiosis, nuclear fusion, mitosis, and differentiation to produce a new micronucleus and a new macronucleus (8). The micronucleus contains one allelic pair of rDNA. The rDNA is excised from the chromosome, converted to a 21-kb head-to-head dimer, and amplified to about 9000 copies as a new macronucleus differentiates (9). Mating *T. thermophila* cells can be transformed with a cloned copy of the micronuclear rDNA bearing a mutation conferring paromomycin resistance (10). This molecule is converted to macronuclear-form rDNA as the new macronucleus develops (Fig. 1*A*) and can completely replace the macronuclear rDNA of the host (3, 10). Extensive mutagenesis of the rDNA has previously been carried out using this system (3, 4, 14). An rDNA construct with a neutral mutation produces transformants that grow well and contain only transformant-type rDNA and rRNA. Most nonfunctional rDNA constructs can also produce transformed lines, due partly to frequent recombination of the rDNA in the macronucleus. These lines contain both host and mutant rDNA and often grow poorly (14).

In choosing an insertion site, at least two factors must be considered. Since nonfunctional rRNAs are often unstable in *T. thermophila* (4, 14), it is important that the antisense insertion not interfere with rRNA function. And since an antisense RNA must interact with mRNAs to be effective, an insertion site on the exterior of the ribosome is desirable. An insertion within a variable region on the exterior of the ribosome might be ideal for preserving both ribosome function and antisense activity. The rDNA vector 5318DN (Fig. 1*A*) contains a 61-bp linker with a unique *Not*I site inserted in the D2 variable region (15) of the *T. thermophila* large subunit rRNA gene and was chosen for this analysis. Ribosomes carrying such an insertion remain fully functional *in vivo* (L. Chen, R.S., and M.-C.Y., unpublished). Portions of the D2 region have been shown to be accessible to chemical attack in *Drosophila melanogaster* ribosomes (16), suggesting that this region is on the exterior of the ribosome.

To determine whether antisense fragments carried within the rRNA can effectively eliminate expression of a wide range of target genes, we have inserted portions of three *T. thermophila* genes into the rDNA vector 5318DN and used these constructs to transform *T. thermophila* cells. In the resulting transformants, antisense activity is assessed by determining target protein levels (for two nonessential genes) or viability of transformed lines (for one essential gene). These data indicate that antisense fragments inserted at a particular site within the rRNA can drastically reduce or eliminate expression of the three target genes tested.

MATERIALS AND METHODS

Culture Conditions, Transformation, and Immobilization Assay. *T. thermophila* strains CU427, CU428, and A*III (which

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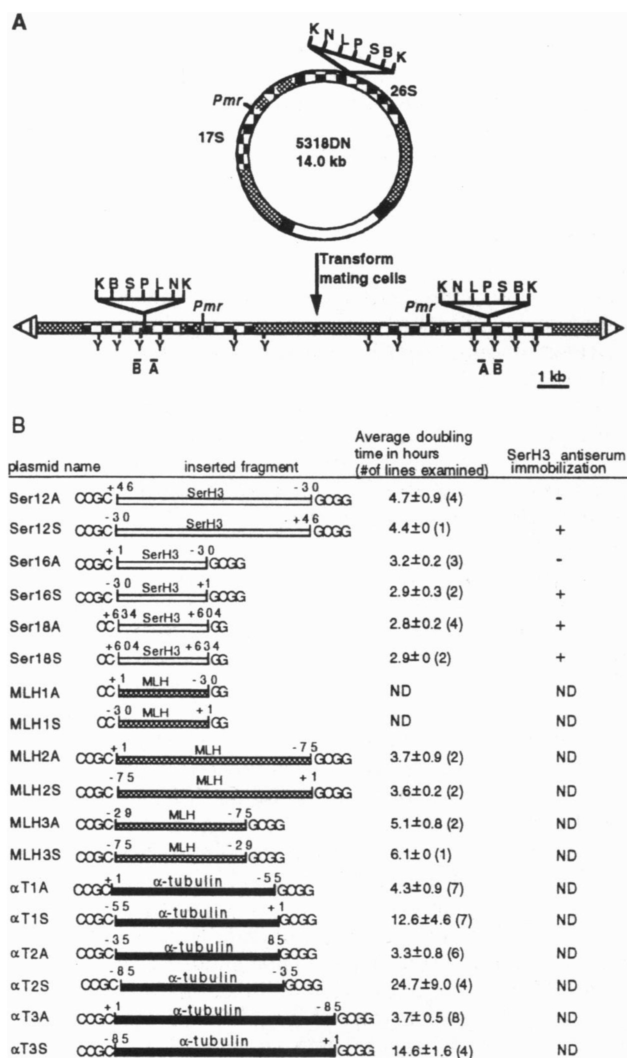

FIG. 1. Transformation and analysis of transformed *T. thermophila* lines. (*A*) A diagram of 5318DN (4), which contains a copy of the micronuclear rDNA, including micronuclear-limited flanking sequences (solid), noncoding portions of the rDNA (shaded); coding regions of the rDNA (checkerboard); and pUC19 sequences (open). The inserted linker at position 5318 in the rDNA sequence (11) has the following sequence: 5′-GGTACCCGCAAAGCGGCCGCGTCGACGGGCCCCCCGGGGTAACCTTTGCGGGTACCCTGAG-3′. K, *Kpn*I; N, *Not*I; L, *Sal*I; P, *Apa*I; S, *Sma*I; B, *Bst*EII; Y, *Sty*I. Telomeres on the palindromic, macronuclear rDNA are indicated by striped arrowheads. Short lines beneath the diagram show the positions of oligonucleotide sequences used as hybridization probes. Their sequences are as follows: A, 5′-TGACAACCCCGTAGTCGGAG-3′; B, 5′-CGTTTCGCGGACGGGTTTTT-3′. *Pmr* indicates the position of a mutation conferring resistance to paromomycin near the the 3′ end of the 17S rRNA gene (12, 13) that is present on the 5318DN vector. (*B*) The diagrams show the sequences inserted into the *Not*I site of 5318DN. The numbers above each diagram denote the position in the sequence of either *SerH3* (open), *MLH* (shaded), or α-tubulin (solid) relative to the initiation codon AUG, with A being +1. Mating *T. thermophila* cells were transformed with the named constructs by use of an alternate selection protocol for the constructs containing α-tubulin fragments. Growth and immobilization assays were performed as described in the *Materials and Methods*. Growth rates for αT1A, αT2A, and αT3A transformants are from the fast-growing transformed lines described in the text. Results of the immobilization assay are indicated as follows: −, not immobilized; +, immobilized; and ND, not determined.

expresses *SerH1*) were obtained from P. Bruns (Cornell University, Ithaca, NY). Strains C2 and *rse*C (which express *SerH4* and *SerL*, respectively) were obtained from F. P. Doerder (Cleveland State University, Cleveland, OH). Mating strains CU427 and CU428 (Cornell University, Ithaca, NY) were transformed with 5–10 μg of plasmid DNA by electroporation (17). Transformants were selected and subsequently grown in enriched peptone media (18) plus paromomycin (130 μg/ml) at 30°C. Control lines have doubling times of 2.5–3.0 hr under similar conditions. An alternate selection protocol was used for αT1A, αT2A, and αT3A transformants (Fig. 1*B*). After transformation, cells (roughly 2×10^6 in 20 ml of 10 mM Tris, pH 7.4) were distributed into 96-well microtiter plates (100 μl per well). After 12 hr at 30°C, 100 μl of media plus paromomycin was added, bringing the final concentration to 30 μg/ml paromomycin. At 48 hr after transformation, 50 μl of media plus paromomycin was added, bringing the final concentration to 130 μg/ml. Immobilization assays were carried out at room temperature in a volume of 20 μl at a 1:40 dilution of D91 antiserum (19) and scored after 1 hr. Control strains expressing *SerH3* or *SerH1* surface antigens were immobilized or not immobilized, respectively, as expected in this assay.

Insertion of Gene Fragments into 5318DN. All fragments were inserted into the *Not*I site of 5318DN (Fig. 1 *A* and *B*). Fragments were amplified from total *T. thermophila* DNA (Ser12, MLH2, MLH3, αT1, αT2, and αT3 constructs) or made by hybridizing single-stranded oligonucleotides (Ser16, Ser18, and MLH1 constructs) and subsequently filling in with Klenow enzyme when necessary (Ser16 constructs). All fragments (except Ser18 and MLH1) were digested with *Not*I before ligation. Primers were designed based on the known sequences of the *SerH3* (20), *MLH* (21), and α-tubulin (22) genes. Their sequences are as follows: Ser12 constructs, 5′-GATGGTACCGCGGCCGCGAAGTTAAGAAATTATTAAGCA-3′; Ser12 and Ser16 constructs, 5′-GATGGTACCGCGGCCGCTAATTCAAACAAAAAATTCAAAAAA-3′; Ser16 constructs, 5′-GATGGTACCGCGGCCGCTTTTTTTTTTTGAATTTTTTGTTTG-3′; Ser18 constructs, 5′-GGCCAAGCAAGACAATCAGCAACTGTCCAAGCATT-3′ and 5′-GGCCAATGCTTGGACAGTTGCTGATTGTCTTGCTT-3′; αT1 constructs, 5′-CGGATGCGGCCGCACTCTTAAGCAGTCCCTCAAGTA-3′; αT1 and αT3 constructs, 5′-CGGATGCGGCCGCTTTCTAACTTTTGATTTGGTTTA-3′; αT3 and αT2 constructs, 5′-CGGATGCGGCCGCTAAAAACAAAAAAAGCAACCTTAAA-3′; αT2 constructs, 5′-CGGATGCGGCCGCTTGAGGGACTGCTTAAGAGT-3′; MLH1 constructs, 5′-GGCCCTTTTTTTTGAAGGATCTTTTTTTTTTTAAGA-3′ and 5′-GGCCTCTTAAAAAAAAAAAGATCCTTCAAAAAAAA-3′; MLH2 constructs, 5′-CGGATGCGGCCGCTTTTTTTTGAAGGATCTTTTTTT-3′; MLH2 and MLH3 constructs, 5′-CGGATGCGGCCGCACAAAATATAATTTATTAAAAGA-3′; and MLH3 constructs, 5′-CGGATGCGGCCGCGAGTCAAATTATTTTTTTTAATA-3′. The structures of all constructs were verified by sequencing the inserted sequence and the immediately flanking regions. The following discrepancies were noted. (*i*) MLH2A contains a 1-bp deletion at position −16, and (*ii*) MLH3S contains two 1-bp deletions (at positions −27 and −49) and one substitution (T for an A at position −57). The numbering is relative to the initiation codon, AUG, where the A is +1.

Western Blot Analysis. To facilitate detection of *SerH3* protein, a protocol was modeled on the initial steps of the purification of the *SerH3* protein (23). About 10^6 cells in mid to late log phase were harvested and washed once in cold 10 mM Tris (pH 7.5). Cell pellets were resuspended in 150 μl of cold 0.1% Triton X-100 in 10 mM Tris (pH 7.5) and vortexed for 20 min at 4°C to lyse. Lysed cells (150 μl) were mixed with 2 μl of 0.1 M HCl and centrifuged for 10 min at 19,350 × *g* at 4°C. To 113 μl of the supernatant, 1.33 μl of 0.1 M NaOH plus 56.5 μl of loading buffer (100 mM Tris, pH 6.8/200 mM DTT/4% SDS/0.2% bromophenol blue/20% glycerol) were added. This sample was boiled for 3 min. Urea (25 mg) was

dissolved in 33 μl of the boiled sample, and 25 μl of this was loaded onto an SDS/12% polyacrylamide gel (24). A 1:1000 dilution of the D91 antiserum (19) was used as the primary antibody. For detection of the *MLH* δ protein, extracts were prepared (25) and loaded onto an SDS/15% polyacrylamide gel. The primary antibody was against the δ protein (26). Western blots were performed using the enhanced chemiluminescence system (Amersham) with minor modifications. The blocking step was performed in 6% casein/1% polyvinylpyrrolidone/0.8% NaCl/75 mM Na HPO_4, pH 7.5 (27). The secondary antibody was anti-rabbit Ig conjugated to horseradish peroxidase (Amersham).

Analysis of DNA and RNA. Southern and Northern blot analyses were performed as described (24, 28). PCR analysis of slow-growing or dying lines was performed as described (14).

RESULTS

To determine whether antisense RNAs inserted within the rRNA could consistently eliminate target gene expression, three *T. thermophila* genes were chosen as targets, *SerH3*, *MLH*, and α-tubulin. Two of these are presumed (*SerH3*) or known (*MLH*; ref. 26) to be nonessential for cell growth, whereas the single α-tubulin gene (29) is very likely to be essential for growth. The *SerH3* gene encodes a surface protein detectable in cells grown between 20°C and 36°C (19). The *MLH* gene encodes linker histone proteins that are localized specifically in the micronucleus. This gene encodes a preprotein that is cleaved to form the β, δ, and γ proteins (21). The α-tubulin gene encodes a protein that is likely to be part of many cellular structures.

DNA fragments containing portions of the *SerH3* and *MLH* genes were inserted into the *Not*I site of 5318DN in both possible orientations (Fig. 1). Three *SerH3* fragments were tested: (*i*) the 31 bases between the 5′ end of the mRNA (20) and the initiation codon, AUG (Ser16A and Ser16S); (*ii*) a 77-bp fragment, including this region plus 46 bases of the neighboring coding region (Ser12A and Ser12S); and (*iii*) a 31-base region near the middle of the coding region (Ser18A and Ser18S). Three fragments of the *MLH* gene were also tested: (*i*) the 76 bases between the 5′ end of the mRNA (21) and the initiation codon (MLH2A and MLH2S); (*ii*) the 31 bases immediately upstream of the initiation codon (MLH1A and MLH1S); and (*iii*) the first 46 bases of the mRNA (MLH3A and MLH3S; Fig. 1*B*). These constructs were used to transform mating *T. thermophila* cells, and the resulting transformants were analyzed.

Target protein levels were assayed by Western blot for both *SerH3* and *MLH*. In addition, the expression of *SerH3* was monitored by a cell immobilization assay (30). *SerH3* protein is undetectable in all lines transformed with the two constructs bearing antisense sequences covering the 5′ end of the *SerH3* mRNA (Ser16A and Ser12A; Fig. 2*A*). Thus, these two constructs show complete inhibition of *SerH3* expression. The third antisense sequence that covers only sequences within the coding region (Ser18A) had no effect. Significantly, all constructs containing *SerH3* sequences in the sense orientation had no detectable effect on protein production (Ser12S, Ser16S, and Ser18S; Fig. 2*A*). Results of the cell immobilization assay (Fig. 1*B*) confirm these results; all Ser16A and Ser12A transformants failed to be immobilized by an antiserum against the *SerH3* protein, whereas transformants from all other constructs and the control, nontransformed cells were immobilized.

A similarly clear antisense effect was seen with the *MLH* gene. All MLH2A transformants (which carried an antisense sequence covering the first 76 bases of the mRNA) produced only trace amounts of δ protein (which is encoded by the *MLH* gene). Transformants from the other two antisense constructs produced normal amounts of δ protein. Again, all three sense constructs had little or no effect on *MLH* protein production (Fig. 2*B*; data not shown for MLH1A and MLH1S transformants).

To determine whether the antisense fragment insertions disturb rDNA or rRNA function, we examined growth rates of transformed lines as well as their rDNA and rRNA composition. Ser16A, Ser16S, Ser18A, and Ser18S transformants contained essentially only rDNA and rRNA with the inserts (Figs. 3*A* and 4*A*). They grew at rates comparable to wild-type lines (Fig. 1*B*). These results show unambiguously that the rDNA and rRNA in these lines are fully functional in spite of the insertions. Transformants of Ser12A, Ser12S, MLH2A, MLH2S, MLH3A, and MLH3S grew slightly slower than wild-type lines (Fig. 1*B*). Only some of these transformed lines contained a small proportion of host rDNA (Fig. 3 *A* and *B*; detectable in long exposures, which are not shown) and rRNA (Fig. 4 *A* and *B*). These inserts may have a slight effect on rRNA function. Thus, in all cases, the rDNA is fully, or nearly

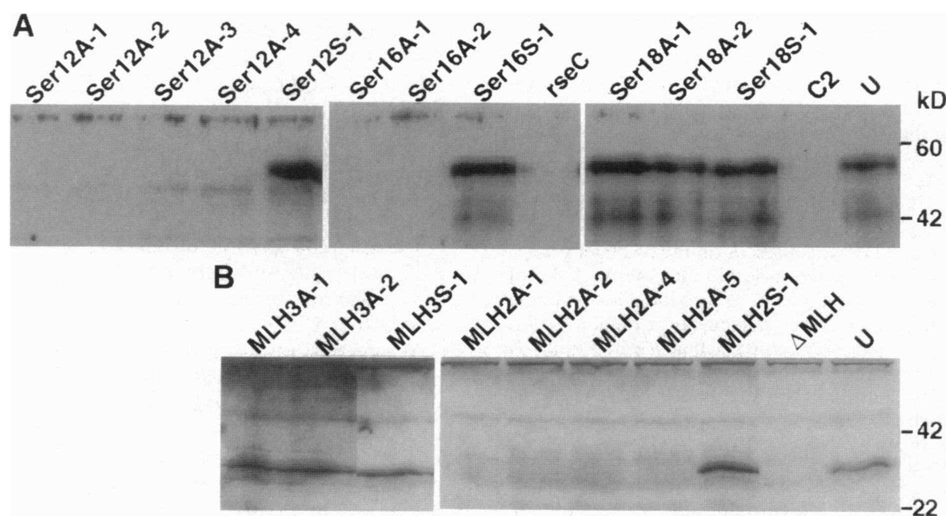

FIG. 2. Western blot analysis of transformed lines. Western blots were performed as described in the text. (*A*) The primary antibody, the D91 antiserum (19), recognizes the *SerH3* protein. All lanes except those labeled U (untransformed CU428 cells) and *rse*C and C2 (lines known to express *SerL* and *SerH4*, respectively; refs. 23, 30) contain protein extracts from transformed lines. The transforming plasmid is indicated followed by a dash and a number indicating the individual transformed line. (*B*) The primary antibody recognizes the δ protein of the *MLH* gene (26). Contents of lanes are indicated as in *A* except that ΔMLH is a line containing an interruption of the *MLH* gene in all the macronuclear copies of this gene (26).

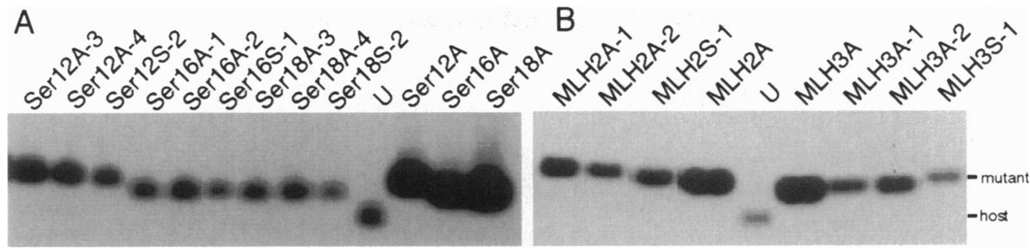

FIG. 3. Southern blot analysis of transformed lines. Total *T. thermophila* DNA was prepared, digested with *Sty*I, and subjected to Southern blot analysis (4) using oligonucleotide A (Fig. 1*A*) as a hybridization probe. (*A*) The left nine lanes contain total *T. thermophila* DNA from transformed lines resulting from the indicated constructs. Numbers following the dashes indicate individual transformed lines. The right four lanes contain total *T. thermophila* DNA from untransformed CU428 (U) or the indicated *Escherichia coli* plasmid constructs (Ser12A, Ser16A, and Ser18A). (*B*) The leftmost and rightmost three lanes contain DNA from transformed lines from the indicated constructs. The middle three lanes contain total *T. thermophila* from untransformed CU428 (U) or the indicated *E. coli* plasmid DNAs (MLH2A and MLH3A).

fully, functional, and the "antisense rRNA" is abundant. These properties help explain the effectiveness of the antisense action.

To determine the level at which the antisense rRNAs act, we examined *SerH3* and *MLH* mRNA levels by Northern blot hybridization. The abundance of *SerH3* message is not significantly reduced in any transformed line. In Ser12A and Ser16A transformants (which produced no detectable *SerH3* protein), *SerH3* mRNA is even more abundant than in untransformed cells (Fig. 4*A*), suggesting that some sort of feedback mechanism might be operating at the level of transcription or mRNA stability. The *MLH* mRNA, however, appears to be less abundant in most transformed lines, regardless of the orientation of the *MLH* insert (Fig. 4*B*). Since there is no correlation between mRNA and protein levels, antisense-bearing rRNAs must prevent translation of the target genes.

Analysis of the α-tubulin gene required a slightly different approach since this single-copy gene is likely to be essential for growth (29, 31). Three DNA fragments from the 5′ untranslated region of the α-tubulin mRNA were inserted into 5318DN in both possible orientations (αT1A, αT1S, αT2A, αT2S, αT3A, and αT3S; Fig. 1*B*), and these constructs were used to transform *T. thermophila*. Data pertaining to only one pair of these constructs (αT1A and αT1S) are presented in detail. Transformants from the other constructs gave similar results.

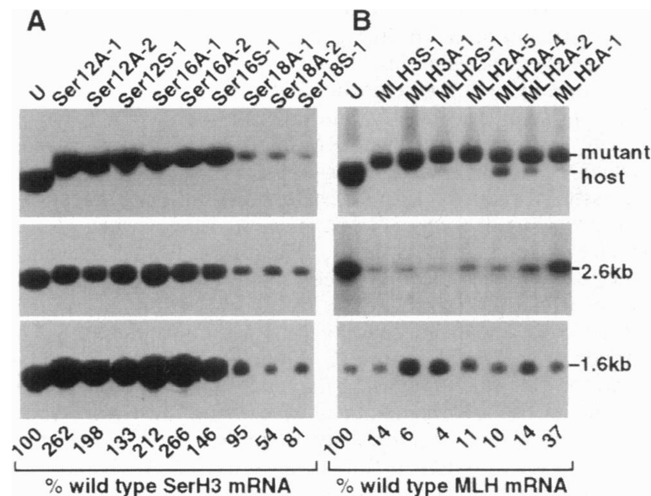

FIG. 4. Northern blot analysis of transformed lines. Total *T. thermophila* RNA was prepared (4) and subjected to Northern blot analysis (24). Sources of RNA in each lane are indicated as described in Fig. 3. Both blots were hybridized with three different probes: oligonucleotide B (*Top*; also shown in Fig. 1*A*); a fragment of the *MLH* gene from nucleotide positions 84 to 1785 (*Middle*); and a fragment of the *SerH3* gene from positions 1167 to 1331 (*Bottom*). (*Top*) The positions of the host and the slightly larger transformant type rRNAs are indicated. (*Middle* and *Bottom*) The approximate sizes of the *MLH* and *SerH3* mRNAs, respectively, are indicated. (*A*) The amount of *SerH3* mRNA in each line relative to a wild-type, untransformed line is indicated below each lane. *MLH* mRNA was used as a control. (*B*) The amount of *MLH* mRNA in each line relative to a wild-type, untransformed line is indicated below each lane. *SerH3* mRNA was used as a control. These data were generated from phosphorimage analysis of the Northern blots shown.

As expected for constructs that might inhibit cell growth, these antisense constructs transformed poorly. Nonetheless, many transformants were obtained when an altered selection protocol was used (*Materials and Methods*). These transformants took 2–4 days longer than 5318DN transformants to appear in the initial selection, grew slowly, and were significantly larger in size and less mobile. Some lines continued to grow very slowly or died (48 out of 160 obtained). PCR analysis of these slow-growing or dying cells showed that, in all cases, a significant proportion of their rDNA contained the α-tubulin insert (Fig. 5). Therefore, these were true transformants. This result indicates that transformation with this antisense construct can be a lethal event.

Many of the αT1A, αT2A, and αT3A transformants remained alive and later began to grow faster (Fig. 1*B*) with near-normal cell morphology. DNA analysis of some of these lines showed that many completely, or almost completely, lacked the α-tubulin gene insertion in their rDNA (Fig. 6; αT1A-1 and -3, αT2A-1 and -3), and the rest contained the insert in only a small proportion (≤15%) of their rDNA. We believe that the antisense inserts were lost because of recombination between the host rDNA and the transforming rDNA, which is known to occur frequently (4). This recombination could generate molecules with the *Pmr* mutation and without the insert, which could confer a selective advantage to the cell. The *Pmr* mutation is about 1.1 kb distant from the insertion site and closer to the palindromic center of the rDNA (see Fig. 1). To determine whether recombinant molecules were present, Southern blots were hybridized to an oligonucleotide specific to the transforming (and not the host) rDNA near its palindromic center. This sequence was abundantly present in

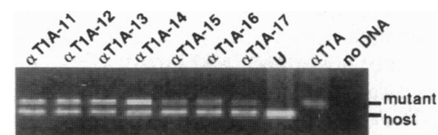

FIG. 5. PCR analysis of slow-growing and dying αT1A transformants. Putative transformants from the original selective microtiter plate were subjected to PCR amplification (14) using oligonucleotides A and B (Fig. 1*A*) as primers. Cells selected were either from wells where all remaining cells subsequently died (αT1A-11 to αT1A-13) or from wells where the remaining cells continued to grow very slowly (doubling time >24 hr; αT1A-14 to αT1A-17). Other lanes are indicated as follows: U, a lysate from untransformed CU428 cells; αT1A, plasmid DNA; no DNA, media from the selection plate without cells.

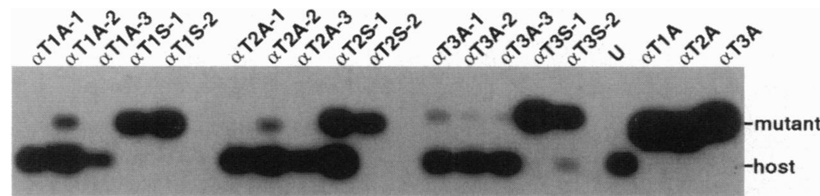

FIG. 6. Southern blot analysis of transformed lines from constructs containing α-tubulin fragments. *T. thermophila* DNA was prepared, digested with *Sty*I, and subjected to Southern blot analysis (4) using oligonucleotide A (Fig. 1) as a hybridization probe. All lanes except the rightmost four contain DNA from the transformed lines indicated above each lane. The right four lanes contain DNA from untransformed *Tetrahymena* (U) and the plasmid vectors indicated.

transformed lines that contained little or no rDNA bearing the α-tubulin insertion (data not shown). These data provide further evidence that antisense rRNA directed against the α-tubulin gene inhibits cell growth when present in sufficient abundance. Thus, by examining the rDNA of both dying and growing transformants, we conclude that antisense rRNA works effectively against the α-tubulin gene and that α-tubulin is an essential gene. These data also suggest that a low proportion of the rDNA (≥15%) bearing the antisense insertion is sufficient to cause lethality. A roughly similar proportion of the rDNA is sufficient to inhibit *SerH3* gene expression (data not shown). As a control, we also analyzed constructs bearing α-tubulin sequences in the sense orientation (αT1S, αT2S, and αT3S). Although these transformants grew slower than normal cells (Fig. 1*B*), most of them contained the expected insert in the vast majority of their rDNA (Fig. 6). Thus, cell lethality is specific to the antisense sequences. The basis of the slow-growth phenotype is unclear. It could be related to the excessive copy number of the 5′ leader sequence of this mRNA, which may titrate out factors necessary for normal cell function.

DISCUSSION

A functional rRNA is stable and abundant and comes into direct contact with mRNAs. Past work has shown that rRNA can tolerate insertion of foreign sequences at certain sites without compromising its function (3, 4). For these reasons, rRNA could be an extraordinary vehicle for carrying a functional RNA species, especially one that acts on mRNAs. We have explored this possibility in the present study by inserting antisense RNAs into a specific site within the *T. thermophila* large subunit rRNA. We have found that rRNA can carry stretches of antisense RNA and remain functional. Such antisense rRNAs can drastically reduce or eliminate target gene expression. The fact that the expression of all three genes tested is drastically reduced strongly supports the idea that an antisense rRNA effect can be robust and consistent.

Although complete gene replacement of the rDNA in *T. thermophila* has facilitated this analysis, it is clearly not required for antisense rRNAs to exert their effects. An effective antisense rRNA targeted against α-tubulin would be expected to be lethal, and our data confirms this expectation (Fig. 5). Viable transformed lines were obtained, but all contained a high proportion of host rDNA and little (≤15%) or no rDNA bearing the antisense α-tubulin insertion (Fig. 6; quantitation not shown). These data suggest that cells containing a greater proportion of this rDNA are not viable, presumably because of the antisense inhibition of α-tubulin expression. In addition, transformed lines containing a 250-bp *SerH3* fragment inserted into 5318DN are viable and show a clear antisense effect, even though only about 17% of their rDNA contains the insertion (data not shown). These data indicate that about 15–20% of the total rRNA can exert effective antisense function. It is still possible that a much smaller proportion of the rRNA may be able to exert similar effects. Thus, these data suggest that antisense rRNAs may be useful in other organisms in which rDNA replacement is not yet possible.

No study has been reported that uses a traditional antisense RNA approach in *T. thermophila*. However, in other systems, high copy number and appropriate intracellular localization are important factors in determining the effectiveness of a given antisense molecule (6, 7), and this is very likely to be true in *T. thermophila* also. It is therefore noteworthy that all, or almost all, detectable protein expression was eliminated in the two cases tested (*SerH3* and *MLH*) with at least one of our antisense constructs. And in the third case (α-tubulin), cell growth was strongly inhibited. Since such complete inhibition is not routinely seen with antisense RNAs, these data suggest that antisense rRNAs can exert robust and consistent antisense effects.

Our data show that target gene expression is inhibited by preventing translation. We can envision at least two mechanisms by which this might happen. In the first, the antisense rRNA hybridizes with the target mRNA in the nucleus and prevents it from being transported to the cytoplasm for translation. There has been at least one documented case in which an antisense RNA probably functioned by this mechanism (32). In the second, the target mRNA hybridizes with the antisense rRNA on a cytoplasmic ribosome and therefore cannot be translated. Future work should be able to distinguish between these possibilities.

Preserving the function of the rRNA is critical for achieving high copy number, stability, and favorable intracellular localization. Chimeric antisense RNAs using tRNAs or small nuclear RNAs as vehicles for antisense RNAs have been successful in reducing target gene expression or viral replication to varying degrees (33, 34). However, these systems have failed to take full advantage of the properties of the host molecules, because they have been rendered nonfunctional by the insertion of the antisense RNAs. Insertion of foreign sequences at most sites in the rRNA compromises rRNA function (refs. 3 and 4; R.S., L. Chen, and M.-C.Y., unpublished observations). During the course of this work, we inserted antisense fragments at five different rDNA sites within four variable regions. Only two of these sites allowed the inserted fragments to function as antisense RNAs. Insertions at two other sites severely compromised rRNA function (data not shown). It would be interesting to define in more detail the sequences or structures necessary for various rRNA variable regions to be fully functional.

Antisense sequences targeted to different portions of the mRNAs had drastically different effects; they either eliminated the target protein or had no effect on its abundance. We found that only antisense fragments covering the 5′ untranslated region of the mRNA were effective, which is consistent with findings in some other systems (6). This contrast is particularly impressive in our system since even the ineffective antisense rRNAs, such as those in Ser18A and MLH3A transformants, are present at a normal rRNA copy number, or roughly 8×10^7 per cell (35). There are at least two possible explanations. First, molecular structures of the mRNA may have rendered some sites more accessible than others. Second,

hybridization of antisense rRNA within a ribosome with the 5′ untranslated region of an mRNA may prevent initiation of translation. If translation is initiated, hybridization of the antisense rRNA with other portions of the mRNA may be disrupted by the process of translation. Thus, only target sites within the 5′ untranslated region could be effectively used by an antisense rRNA.

The abundance, stability, and favorable intracellular location of rRNA make it a unique and highly attractive vehicle for RNAs with specific biological activities, especially those that act on mRNAs. In *T. thermophila*, it should be possible to use this system to analyze the function of cloned genes, thus offering an alternative method to the gene knockout system (26). The antisense rRNA method may be advantageous in some situations, since it (*i*) affects gene expression at the level of translation rather than by eliminating the gene; (*ii*) could affect both parental and zygotic gene function throughout the process of conjugation; (*iii*) requires no growth period after transformation to accomplish complete, or almost complete, inhibition of gene expression; and (*iv*) could affect the expression of multigene families if they share identical antisense target sequences. This method may also make it possible to clone genes by their null or hypomorphic phenotypes, which would represent a significant technical advance for this biological system.

It is likely that this method may also be applied to other systems. Although total rDNA replacement is not yet possible in organisms other than *T. thermophila* and *Saccharomyces cerevisiae*, it is clearly not required for the method to work since as little as about 15% of the rRNA (and possibly much less) is sufficient to eliminate target gene expression. The fact that rRNA can be an effective carrier for antisense RNAs suggests that it may also be used for carrying other RNAs that could affect cell function, such as small, trans-acting ribozymes designed to cleave specific mRNAs (36). Because of the enzymatic nature of ribozymes, ribozyme-bearing rRNAs may be required in lower copy numbers than antisense rRNAs and, thus, may be even more generally useful.

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