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Phenotypic Effects of Targeted Mutations in the Small Subunit rRNA Gene of *Tetrahymena thermophila*

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***Tetrahymena thermophila* is an ideal organism with which to study functional aspects of the rRNAs in vivo since the somatic rRNA genes of *T. thermophila* can be totally replaced by cloned copies introduced via microinjection. In this study, we made small insertions into seven sites within the small subunit rRNA gene and observed their phenotypic effects on transformed cells. Two mutated genes coding for rRNA (rDNAs), both of which bear insertions in highly conserved sequences, failed to transform and are therefore believed to produce nonfunctional rRNAs. Three other altered rDNAs produce functional rRNAs that can substitute for most or all of the cellular rRNA. Two of these bear insertions in highly variable regions, and, surprisingly, the other has an insertion in a region that is well conserved for both sequence and secondary structure among eucaryotes. In addition, two other insertions appear to destabilize rRNAs that contain them. Our findings make predictions concerning the positions of some of these sites within the tertiary structure of the small ribosomal subunit and thus serve as an in vivo test of the existing tertiary structure models for the small subunit rRNA. Our results are in good agreement with expectations based on sequence comparison and in vitro work.**

Although it is generally acknowledged that rRNAs play a crucial functional role in translation, the molecular details of this process are poorly understood. The physical structure of small subunit (SSU) rRNAs has been extensively investigated. Primary sequences from at least 275 organisms have been reported (25). A model for secondary structure of the SSU rRNA (26) has been confirmed by biochemical evidence (18) and the accumulation of additional sequence data (25). Sequencing results have revealed many regions of high sequence conservation, which presumably play an important role in ribosome structure or function. Several models for the tertiary structure of the small ribosomal subunit have been proposed on the basis of different sets of data (3, 24, 27, 38). Genetic studies have identified specific bases that are important for ribosome assembly (43), subunit association (32), and translational initiation, accuracy, and termination (9, 10, 20, 41, 44). In vitro studies have identified bases that can be cross-linked to tRNA (33). Bases that can be protected from chemical or enzymatic attack by the binding of tRNAs, message, and the large ribosomal subunit have also been identified (16, 17).

Genetic studies with rRNA genes (rDNA) have been difficult, since the rDNA is present in multiple copies in most organisms. Two exceptions to this are *Tetrahymena thermophila* (46) and *Halobacterium halobium* (15). Most classical genetic studies have been confined to mitochondrial genomes, which often contain only one copy of the rDNA. In recent years, problems inherent in this type of study have been circumvented in some systems (9, 12, 23, 40, 41, 43). One such system is *T. thermophila*, in which the resident rDNA can be replaced with rDNA introduced via microinjection (49). Since the present understanding of the molecular basis of rRNA function is very limited, we have performed a mutagenesis study of the SSU rRNA, hoping to uncover interesting but previously uninvestigated regions. We have examined several aspects of the phenotypes of cells transformed with each of 10 altered rDNA constructs bearing insertions at seven different sites within the SSU rRNA gene.

The system we have used possesses some special features that are relevant to the present study. *T. thermophila* has two nuclei: a micronucleus, or germline nucleus, and a macronucleus, or somatic nucleus, in which virtually all transcription takes place (11). The micronucleus contains five pairs of chromosomes and only one pair of rRNA genes (46), one on each copy of chromosome II (4, 5). The macronucleus is derived from a replicate of the micronuclear genome and contains an amplified genome with about 85% the sequence complexity of the micronucleus (47). During vegetative growth, the macronucleus divides amitotically, which can lead to the sorting out of different alleles of the same gene into different progeny cells (28, 29). During conjugation, cells of different mating types fuse, their micronuclei undergo meiosis, and their macronuclei are degraded. Each mating partner forms a new, diploid micronucleus through the fusion of two haploid meiotic nuclei, one donated by each parent. New macronuclei are formed from replicates of the new micronuclei. During this process, the genome goes through a specific series of cleavages, deletions, telomere additions, and gene amplification events. The chromosomes are fragmented into molecules with an average size of 600 kb, and these are amplified to about 45 copies per macronucleus (45). The rDNA is converted from a single gene copy integrated into a chromosome to an extrachromosomal palindromic molecule 21 kb in size (Fig. 1). This molecule is present in about 9,000 copies per macronucleus (48).

An rDNA-based transformation system (42) facilitates the present work. It utilizes two genetic markers, one of which is a single base change near the 3′ end of the 17S rRNA gene (*Pmr*) that confers paromomycin resistance (pm-r) in *T. thermophila* (5, 36). Another arises from two inbreeding lines of *T. thermophila*, the C3 type and the B type. The C3 type rDNA is preferentially maintained in the macronuclei of cells containing both C3 and B type rDNA, usually resulting in complete loss of B type rDNA from the macronucleus (13, 30, 42). When developing macronuclei of mating cells that

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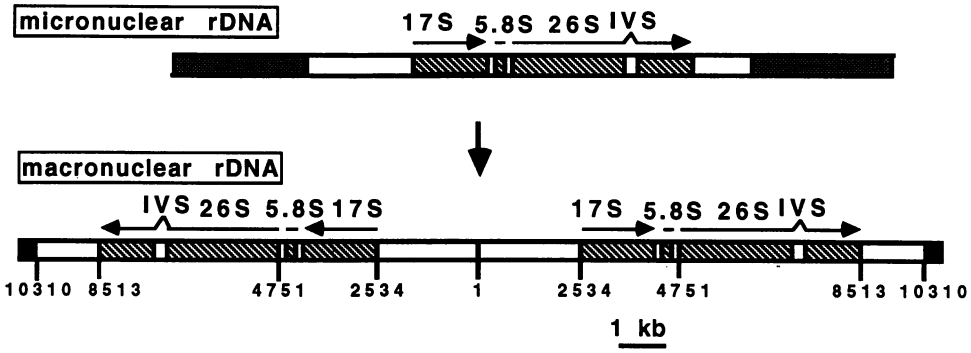

FIG. 1. Conversion of micronuclear rDNA to macronuclear rDNA. The upper diagram represents the single copy of the rDNA found on micronuclear chromosome II. The lower diagram represents the 21-kb palindromic macronuclear rDNA. Regions on the DNA are indicated as follows: shaded, micronuclear DNA that is not included in the macronuclear rDNA molecule; open, noncoding regions included in the macronuclear rDNA; hatched, coding regions of the rDNA; solid, telomeres. The names of the rRNA genes and the direction of transcription are indicated above each diagram. IVS means intervening sequence, which is spliced out posttranscriptionally. Below the lower diagram are position numbers relative to the center of the palindromic macronuclear rDNA. This numbering system is used throughout this paper to refer to positions in the rDNA and rRNA.

are B type and paromomycin sensitive (pm-s) are injected with a cloned copy of the micronuclear version of *Pmr*, C3 type rDNA, pm-r transformants are obtained at a frequency of between 0.5 and 10.0% of the injected cells. Most of these pm-r transformants have completely replaced their host rDNA with an injected rDNA, which has been accurately processed into a palindromic, macronuclear form rDNA (40, 49).

In the present study, we have introduced small insertions into seven sites within the SSU rRNA gene and have used these mutated rDNAs to transform wild-type cells. In most cases, pm-r transformants were obtained, and several aspects of the phenotypes of these transformed lines were observed. Our results suggest that some mutated rRNAs are fully functional and others are nonfunctional. In some cases, these data can be interpreted as being supportive or nonsupportive of the existing models for the tertiary structure of the SSU.

MATERIALS AND METHODS

Strains, culture conditions, and transformation. The strains CU427 (*Chx/Chx* [VI, cy-s]) and CU428 (*Mpr/Mpr* [VII, mp-s]) from the inbreeding line B were obtained from Peter Bruns. Cells were grown in enriched peptone media (6) with or without paromomycin (100 μg/ml; obtained as capsules of paromomycin sulfate [Humatin] from Parke-Davis). CU427 and CU428 were mated and subjected to microinjection as previously described (42, 49).

Construction of altered rDNAs. The construction of altered rDNAs is diagrammed in Fig. 2. The plasmid 947-400 (identical to D5-400 [50]), which contains a micronuclear copy of the *Tetrahymena* rDNA as well as a gene conferring ampicillin resistance in bacteria, was partially digested with *Hin*fI or *Dde*I to yield molecules that were uncut or cut only once. Conditions for such digestions were determined empirically with the work of Parker et al. (31) as a guide. The plasmid SQ60 was constructed by replacing the polylinker of pHSS6 (34), which contains a kanamycin resistance gene, with one formed from two synthetic oligonucleotides. The sequences of these synthetic oligonucleotides are 5′GGCCGCTTTAAA GGTTACCCCGGGGGGCCCGTCGACGC3′ and 5′GGCC GCGTCGACGGGCCCCCCGGGGTAACCTTTAAA GC3′. SQ60 was digested with *Dra*I, creating a blunt end

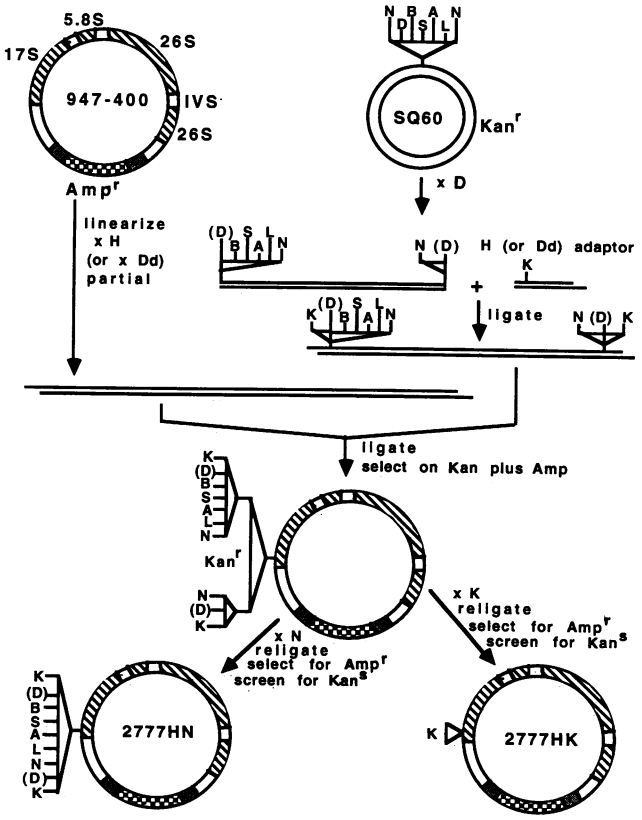

FIG. 2. Construction of the linker insertions. This series of steps is described in detail in Materials and Methods. Different regions on these plasmid DNAs are indicated as follows: hatched, coding regions of the rDNA; open, noncoding regions included in the macronuclear rDNA (except in the plasmid SQ60, where they represent vector sequences); shaded, micronuclear sequences that are not present in macronuclear rDNA; checkered, pUC19 sequences containing the ampicillin resistance (Amp^r) gene. The following abbreviations indicate restriction sites: H, *Hin*fI; Dd, *Dde*I; N, *Not*I; D, *Dra*I; B, *Bst*EII; S, *Sma*I; A, *Apa*I; L, *Sal*I; and K, *Kpn*I. (D) indicates one-half of a *Dra*I site at which cleavage has occurred. Kan^r, kanamycin resistance gene.

within the polylinker. Two synthetic adaptor molecules containing a *Kpn*I site were made, which were blunt on one end and had an overhang matching that left by *Hin*fI or *Dde*I on the other end. The sequences of the oligonucleotides comprising the *Hin*fI adaptor are 5'ANTCGGTACCCGC3' and 5'GCGGGTACCG3'. The sequences of the oligonucleotides comprising the *Dde*I adaptor are 5'TNAGGGTAC CCGC3' and 5'GCGGGTACCC3'. The N in these oligonucleotides represents an equimolar mixture of the four deoxynucleotides. These adaptors were ligated onto the linearized, blunt-ended SQ60. These molecules were then ligated to the partially digested 947-400, and transformants of the bacterial strain DH1 (8) were selected on kanamycin plus ampicillin. DNA from these transformants was then digested with either *Not*I or *Kpn*I, religated, and retransformed to yield ampicillin-resistant and kanamycin-sensitive transformants that had either a 61-bp or an 11-bp insertion at various *Dde*I and *Hin*fI sites. The positions of the insertions were mapped by restriction digestion.

Isolation and analysis of *Tetrahymena* DNA. Total *Tetrahymena* DNA was isolated as previously described (1). Southern blotting was performed essentially as described previously (35). Oligonucleotides were synthesized with an Applied Biosystems oligonucleotide synthesizer and were labeled by addition of ^{32}P-labeled phosphate to the 5' ends (14).

Isolation and analysis of *Tetrahymena* RNA. *Tetrahymena* RNA was isolated as previously described (40) with the following changes. Log-phase cultures of 10 to 15 ml (rather than 100 ml) were harvested, washed, resuspended in 0.5 ml of 7 M urea–2% sodium dodecyl sulfate–20 mM NaOAc–10 mM EDTA (pH 7.0), quick-frozen in a dry ice-ethanol bath, and stored at −80°C prior to completing the remainder of the preparation.

Northern (RNA) blot analysis was performed as previously described (40). Hybridization of short oligomers (*Hin*fI linker and *Dde*I linker; see Fig. 6) to Northern blots was performed as follows. Prehybridization and hybridization were carried out in 15% formamide–5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–25 mM $NaPO_4$ (pH 6.8)–50 μg of salmon sperm DNA per ml–5× Denhardt's solution (2). Blots were incubated for 20 min at 26°C prior to the addition of the labeled oligonucleotide. After addition of the labeled oligonucleotide, the hybridization was heated to 50°C for 10 min and then was slowly cooled to 26°C and was allowed to hybridize at this temperature overnight.

Reverse transcription followed by polymerase chain reaction (PCR) to amplify the segment of interest was performed in the following way. Prior to reverse transcription, RNA (2 μg) was treated with RNase-free DNase (1 U; Promega) in a total volume of 20 μl in a buffer with the following final composition: 10 mM Tris-HCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM dithiothreitol, 50 μg of bovine serum albumin (BSA) per ml, and 100 mM KCl (pH 8). This mixture was incubated for 10 min at 37°C, followed by 10 min at 65°C, and it was then precipitated with ethanol. About 1 μg of total *Tetrahymena* RNA, 0.5 μl of RNAsin (Promega), 200 U of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories), 100 pmol of random hexamers (Pharmacia), 10 nmol of deoxynucleoside triphosphates, and 0.1 mg of BSA per ml were combined in a total volume of 10 μl with a final concentration of 50 mM KCl–20 mM Tris-HCl (pH 8.4)–3 mM $MgCl_2$. This mixture was incubated for 10 min at room temperature, 45 min at 50°C, and then 5 min at 95°C. In some cases, about 50 ng of a specific primer complementary to the rRNA was used in place of the random hexamers. Primers for PCR (about 50 ng each) and *Taq* polymerase (about 0.2 U; Cetus) in a buffer with a final concentration of 50 mM KCl–20 mM Tris-HCl (pH 8.4)–1.13 mM $MgCl_2$–0.1 mg of gelatin per ml and a total volume of 40 μl were then added to the reverse transcription reaction mixture (10 μl). In cases where a specific primer complementary to the rRNA had already been added to the reverse transcription reaction mixture, only the one remaining primer necessary for the PCR was added. This mixture was then amplified for 25 to 30 cycles.

RESULTS

Linker insertion constructs. Altered rDNAs with small insertions within the SSU rRNA gene were made by partial digestion with either *Hin*fI or *Dde*I as explained in detail in Materials and Methods and diagrammed in Fig. 2. This method produced rDNAs with either of two types of inserted linkers (61 or 11 bp in length) at seven different sites within the SSU rRNA gene. These sites are spread throughout the SSU rRNA gene and represent a variety of kinds of regions with respect to conservation of sequence and secondary structure. This collection of sites offers opportunities to explore regions about which some functional information is available as well as regions about which little is presently known. The positions of these sites within the *Tetrahymena* SSU rRNA secondary structure (36) are shown in Fig. 3.

There is some information that points to possible functional roles for the regions of the rRNA in which the seven sites fall. Two of the insertion sites, 3058 and 3185 (the numbers refer to the position with respect to the middle of the *Tetrahymena* macronuclear rDNA palindrome [Fig. 1]), are in areas in which the sequence and secondary structure of the SSU rRNA are not well conserved. It is likely that insertions might be tolerated at these two positions, although this has not been the case for every insertion in every variable region that has been tested (21, 40). In contrast, sites 2919 and 3694 fall in very highly conserved single-stranded regions, which thus might play some role in ribosome structure or function. Insertions at these two sites might impair or obliterate rRNA function. Although site 2777 lies within the V2 variable region (25), sequence and secondary structure are somewhat conserved in this area among eucaryotes. For example, among 20 eucaryotic SSU rRNA sequences aligned in the study by Neefs et al. (25), only 1 does not have this secondary structure and 13 have a *Hin*fI site in an identical location. Nearby bases show S20-dependent enhancement of and protection from chemical attack in *Escherichia coli* ribosomes (37). It is difficult to predict the effects of an insertion in this area. Position 2822 is within a double-stranded stem that is conserved for length and is very resistant to chemical attack in *E. coli* ribosomes (18), suggesting that this helix might be located at an interior position within the ribosome. On this basis, it seems likely that insertions at site 2822 would not be tolerated. The 3883 site lies within a stretch of sequence that is well conserved among eucaryotes and is adjacent to an area that is variable in size and sequence. Insertions at this site might have adverse effects on rRNA function.

Our constructs were named according to the position of the insertion within the rDNA, the restriction site into which the insertion was made, and the type of insertion. For example, 2777HK refers to a construct which has an 11-bp insertion (signified K) at the *Hin*fI site (signified H) at position 2777 in the rDNA. The name 3058DN refers to a

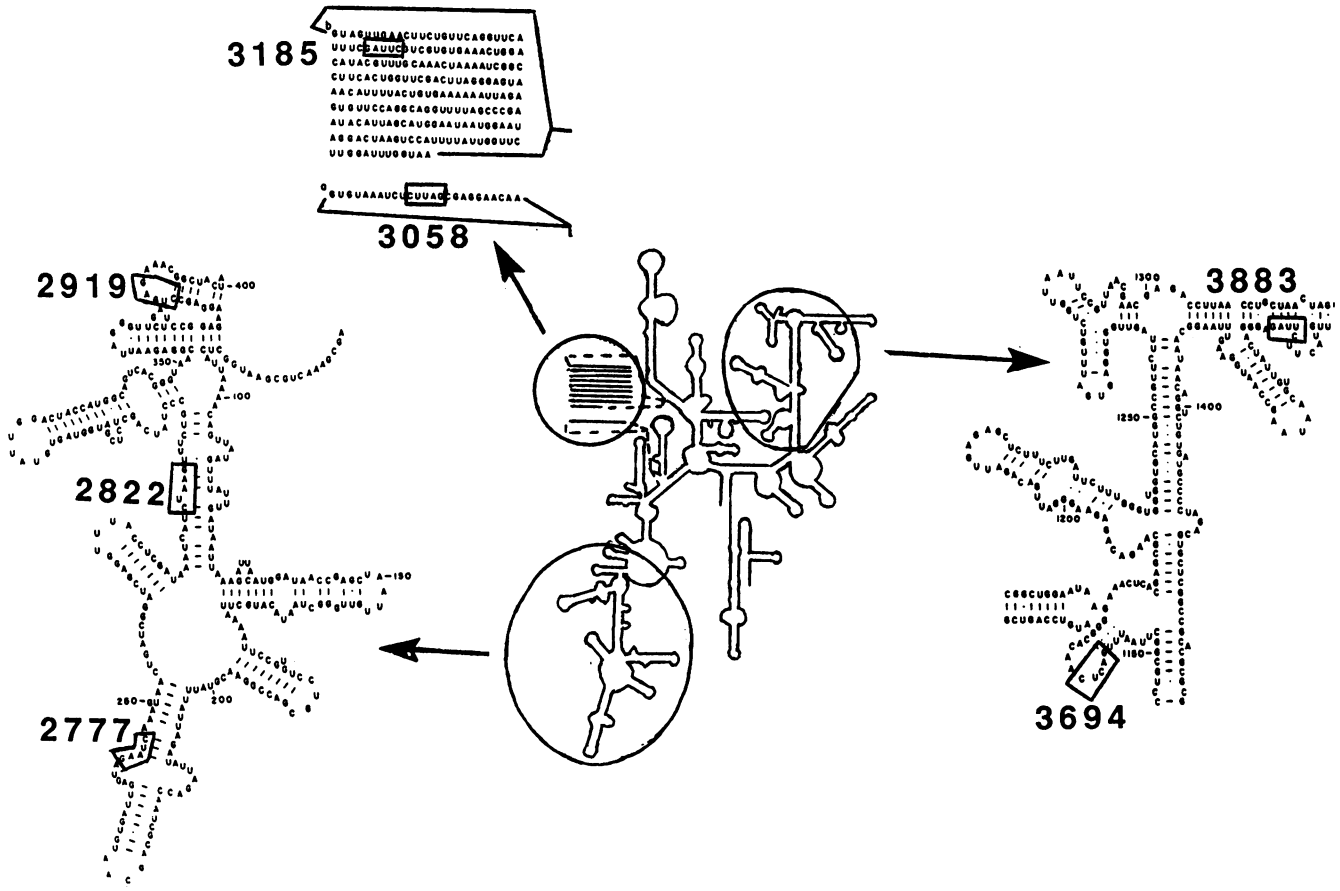

FIG. 3. Positions of the insertions within the secondary structure of the SSU rRNA. In the center of this figure is a representation of the secondary structure of the *Tetrahymena* SSU rRNA (33). The circled areas are shown enlarged. The restriction sites into which insertions were made are boxed and are indicated with a number. The number represents the distance of the site from the center of the macronuclear rDNA molecule (see Fig. 1).

construct that has a 61-bp insertion (signified N) at the *Dde*I site (signified D) at position 3058 in the rDNA.

Growth phenotype of *Tetrahymena* transformants. In order to test their transforming abilities and observe the phenotype of transformed cells, the constructs described above were injected into mating *Tetrahymena* cells. Three of the constructs failed to transform. In all other cases, pm-r transformants were obtained at frequencies comparable to those obtained with a wild-type rDNA clone. These results are summarized in Table 1.

The transformants were monitored for their growth rate at 30°C. Control transformants, resulting from injection of 947-400, grew with doubling times of 3 to 3.5 h in media containing paromomycin at 100 μg/ml. Transformants resulting from injection of 3883DN were initially able to grow in media with 50 μg of paromomycin per ml but were inviable when maintained for long periods of time under drug selection. Transformants resulting from injection of all other constructs grew with doubling times between 3.5 and 6.0 h under the same conditions (Table 1). All transformants (except 3883DN transformants) were able to grow at low temperature (18°C) under drug selection.

Analysis of the rDNA in transformed cells. Although most cell lines transformed with unmutated 947-400 contain only injected type rDNA (49), this might not be the case for cells transformed with mutated constructs. Since a macronucleus contains on average 9,000 rDNA molecules which are able to assort during division, a transformant could contain a mixture of host and injected rDNA, which might provide information regarding the properties of the injected rDNA. DNA was extracted from transformant lines grown in media with paromomycin and analyzed by Southern blot. The DNA was digested with *Kpn*I (which cleaves at the insertion site but at no site within the unaltered rDNA) as well as either *Bst*BI or *Eco*RV (both of which produce a piece approximately 2 kb in size that includes the entire SSU rRNA coding region). Either of two oligonucleotides containing sequences within the SSU rRNA was used as a hybridization probe. Thus, in all cases, the host rDNA will produce a band of about 2 kb in size and the injected rDNA will produce a smaller band, the exact size of which depends on the site of the insertion and the hybridization probe used. A restriction map of the region is shown in Fig. 4. The Southern blots are shown in Fig. 5, and this information is tabulated in Table 1.

All cell lines transformed by 3058DN and 3185HK contain almost exclusively rDNA of the injected type. By this criterion, these rDNA clones behaved like unaltered 947-400. Since transformants resulting from these constructs also grow as well as wild-type transformants, it seems that the insertions we have made in these two constructs have little or no effect on rRNA function.

In contrast, all 2777HK, 2822DK, 2822DN, 3883DK, and

TABLE 1. Results of transformation experiments with *Tetrahymena* rDNA

Plasmid[a]	No. of transformants	Transformation frequency (%)[b]	Replacement of rDNA[c]	Doubling time[d]	Injected type rRNA[e]
3058DN	1	0.3	+++	4	+++
3185HK	8	3.3	+++	3.5	+++
3883DK	3	1.0	+++	6	+++
2777HK	4	1.5	+	4–6	–
2822DK	5	2.0	+	4	–
2822DN	7	3.2	+	5.4	–
3883DN	2	0.5	+	ND[f]	ND
2919DK	0	<0.4	ND	ND	ND
2919DN	0	<0.1	ND	ND	ND
3694HK	0	<0.3	ND	ND	ND

[a] The number preceding the letters indicates the position in the *Tetrahymena* rDNA sequence where the insertion occurs. The first letter indicates the restriction site into which the insertion was made: H, *Hin*fI; D, *Dde*I. The second letter indicates the nature of the linker insertion: K, an 11-bp insertion; N, a 61-bp insertion. The sequences of these insertions are provided in the text.

[b] Values are numbers of transformants obtained divided by the number of cells injected.

[c] + indicates partial replacement of the host by the injected rDNA where the injected rDNA comprises between 30 and 70% of the rDNA present in the cells. +++ indicates total (or almost total) replacement of the host by the injected rDNA.

[d] Doubling time (in hours) at 30°C in media with paromomycin at 100 μg/ml. Wild-type cells (i.e., pm-s cells transformed with an unaltered pm-r rDNA) grow with a doubling time of 3 to 3.5 h under the same conditions.

[e] +++ indicates that the majority of rRNA present (as detected by reverse transcription followed by PCR) is of the injected type. – indicates that almost none of the rRNA present is of the injected type.

[f] ND, not done.

3883DN transformants contained both host and injected type rDNA molecules. In order to assess the phenotype of transformant lines that contained only injected rDNA, we subcloned some of these transformant lines. Since the division of the macronucleus in *T. thermophila* species is amitotic, it is possible to obtain individual cells within a line with different proportions of a given allele when more than one allele is present. This assortment can ultimately result in lines with only one allele (28, 29). In the case of the 3883DK transformants, which initially contained minor amounts of host rDNA, we recovered several subclones that contained almost exclusively injected type rDNA (Fig. 5, panel 3883DK, lanes A_1, A_3, and B_4).

The situations with transformed lines resulting from injection of 2777HK and 2822DK were different. The initial transformants appeared to contain ≤50% injected type rDNA. We repeated the injection of these two constructs at higher DNA concentrations and at a slightly earlier time during the development of the macronucleus (when fewer copies of host rDNA are present). The six transformants obtained with 2777HK and the four transformants obtained with 2822DK again had approximately 50% injected type rDNA (data not shown). A total of 37 subclones isolated from these 10 transformants were examined, and all had about the same proportion of injected type rDNA (data not shown). From these results, we conclude that 2777HK and 2822DK are not able to completely replace the host rDNA. We did not subclone transformant lines from 2822DN or 3883DN.

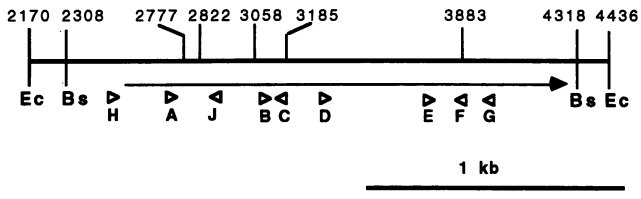

FIG. 4. Detailed map of the SSU rRNA gene. The horizontal arrow beneath the horizontal line indicates the extent of the coding region of the SSU rRNA gene. Above the horizontal line, sites into which insertions were made are indicated. Restriction sites used in DNA analysis are indicated with a number above the line and two letters below: Bs, *Bst*BI; Ec, *Eco*RV. The open arrowheads beneath the horizontal arrow show the positions and orientations of oligonucleotides used as hybridization probes and PCR primers. The sequences of A, B, C, D, E, F, G, H, and J are 5'TGTCCTGCGAC CGGAACGTA3', 5'CCAGCTCCAATAGCGTATAT3', 5'ACCCA ACTACGAGCTTTTTAACT3', 5'ACCTAGCATGGAATAATGG AATA3', 5'GACAGAGAAGGGATTGACAG3', 5'GTCCCTCTA AGAAGTACA3', 5'GGAAATACTTTTTGCGCCAG3', 5'AATG AGCAAGCTGTTGGAAG3', and 5'CTGCCATGGTAGTCCAAT AC3', respectively.

The six transformants obtained with 2777HK and the four transformants obtained with 2822DK again had approximately 50% injected type rDNA (data not shown). A total of 37 subclones isolated from these 10 transformants were examined, and all had about the same proportion of injected type rDNA (data not shown). From these results, we conclude that 2777HK and 2822DK are not able to completely replace the host rDNA. We did not subclone transformant lines from 2822DN or 3883DN.

Three constructs examined here, 2919DK, 2919DN, and 3694HK, failed to produce transformants. In order to rule out a technical explanation for this result, 2919DK and 3694HK were coinjected with another altered construct that failed to transform as well as one that transformed but failed to fully replace the host rDNA. In the six transformants recovered in the case of 2919DK and the five transformants recovered in the case of 3694HK, no rDNA bearing insertions at either site 2919 or site 3694 was detected (data not shown). We had previously found that when several different mutated rDNAs that, alone, are capable of transformation are coinjected, all of these mutated rDNAs are represented among some of the resulting transformants (39). Thus, it is very unlikely that the inability of 2919DK and 3694HK to transform is due to a technical failure. The construct 2919DN, which also failed to produce transformants, was not included in a coinjection experiment on the assumption that if a small insertion at position 2919 prevented transformation, so would a larger insertion at the same site.

Analysis of the rRNA in transformants. Because the injected rDNAs were detected in all transformants, we expected that rRNA bearing the same insertion should also be present. Whole-cell RNA was extracted from the transformants and was subjected to Northern blot analysis with oligonucleotides complementary to the inserted sequences as hybridization probes. RNAs from 3058DN, 3185HK, and 3883DK transformants hybridize strongly with the inserted sequence (Fig. 6D, E, and F). This is expected, since these transformants contain almost entirely injected type rDNA. In contrast, 2777HK, 2822DK, and 2822DN transformants

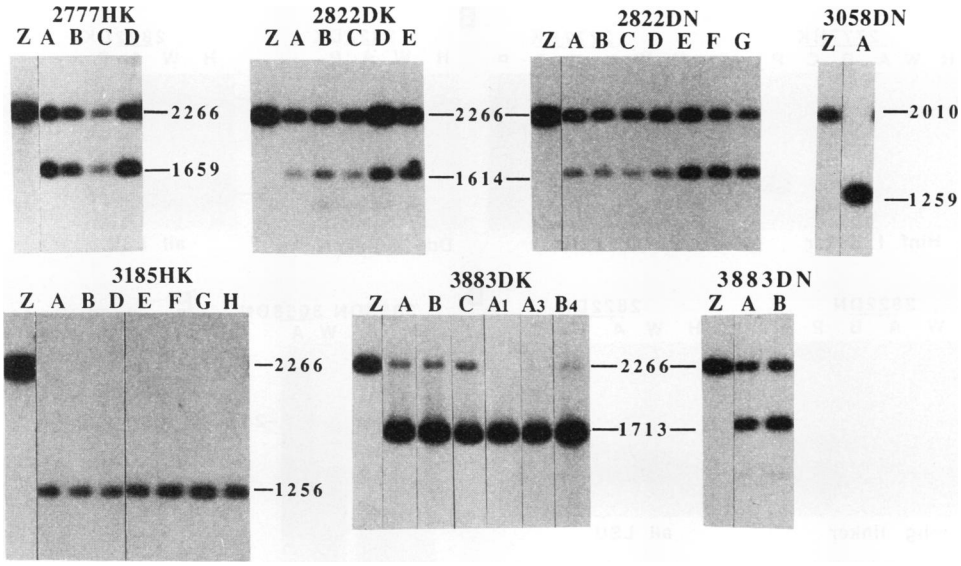

FIG. 5. Species of rDNA present in transformants. DNAs were extracted from transformed lines resulting from injection of the construct named at the top of each panel. In panels 2822DK, 2822DN, 3185HK, 3883DK, and 3883DN, the DNAs were digested with *Eco*RV and *Kpn*I (which cuts only at the site of the insertion), and oligonucleotide D (Fig. 4) was used as a hybridization probe. In panel 3058DN, the DNAs were digested with *Bst*BI and *Kpn*I, and oligonucleotide D (Fig. 4) was used as a hybridization probe. In panel 2777HK, the DNAs were digested with *Eco*RV and *Kpn*I, and oligonucleotide C (Fig. 4) was used as a hybridization probe. Lane Z in each panel contains DNA from the untransformed strain CU427. Lanes A to H contain DNA from individual transformed lines. In the 3883DK panel, lanes A_1 and A_3 contain DNAs from subcloned lines of transformant A, and lane B_4 contains DNA from a subcloned line from transformant B. The numbers next to the bands indicate the estimated sizes (in base pairs) of these fragments. The upper bands result from rDNA without insertions, and the lower bands result from rDNA that contains insertions.

contain little or no injected type rRNA (Fig. 6A, B, and C), in spite of the fact that they contain substantial quantities of injected type rDNA. This result suggests that injected type rRNAs in these transformants are inefficiently transcribed and/or preferentially degraded.

It is possible that hybridization may be inefficient in some regions of the rRNA, resulting in a failure to detect injected type rRNA by hybridization to Northern blots. In addition, this method does not simultaneously detect the presence of host and injected type rRNAs. In order to assess the relative amounts of host and injected rRNA present in the transformants, whole-cell RNAs were analyzed by reverse transcription followed by PCR to amplify the segments of cDNA spanning the insertion site. The PCR products were analyzed by gel electrophoresis (after cleavage with *Kpn*I, which cuts only at the insertion site, when necessary) to reveal host and injected type rRNAs. In order to control for possible DNA contamination, all RNAs tested were subjected to PCR amplification without prior reverse transcription. In all cases, no band was detected without reverse transcription (data not shown).

It is clear that the transformants from 3058DN, 3185HK and 3883DK tested contain almost exclusively rRNA of the injected type (Fig. 7D, E, and F). It is also clear that little or no injected type rRNA is present in the 2777HK, 2822DK, and 2822DN transformants tested (Fig. 7A, B, and C). Our unpublished data suggest that PCR can detect DNA species comprising 5% of the total but cannot detect species comprising 1% of the total. Thus, our results show that 3058DN, 3185HK, and 3883DK transformants contain almost exclusively injected type rRNA and strengthen the conclusion that these rRNAs function well in the cell. They also show that 2777HK, 2822DK, and 2822DN transformants contain almost exclusively host type rRNA, in spite of the fact that they contain substantial quantities of injected type rDNA.

The reason for the absence of these rRNAs is not clear. It is probable that the rRNA is transcribed and rapidly degraded. The 35S rRNA precursor species (19) is barely detectable in Northern blots of RNA from wild-type strains (data not shown). To determine whether these species were present, RNAs from the transformants mentioned above were subjected to reverse transcription followed by PCR, with an oligomer with sequences from about 50 bp upstream of the SSU rRNA coding region and one with sequences from about 50 bp downstream of site 2822 (oligomers H and J, Fig. 4) as PCR primers. These primers will detect only precursor species since mature species are cleaved at the start of the SSU coding region (19). The resulting PCR products can also be digested with *Kpn*I to detect the presence of the inserted sequence when necessary. The results (Fig. 8) show that, in addition to the host sequence (0.4-kb band), minor amounts of injected type rRNA precursor species can be detected in the transformant line 2777HK-A (0.3-kb band). A longer exposure reveals an even smaller amount in the transformant line 2777HK-B (data not shown). On the other hand, no injected rRNA species were detected in either 2822DK-A or 2822DN-A RNA (0.35- or 0.46-kb band). When amplified without prior reverse transcription, no bands were detected in any of these RNA samples (data not shown). These data indicate that 2777HK rDNA is transcribed and that the resulting rRNA is present in low abundance in precursor form in 2777HK transformants.

Detection of recombinant rDNAs. Since the *Pmr* mutation was linked to the insertion on the rDNA used for transformation, we wondered how 2777HK, 2822DK, and 2822DN

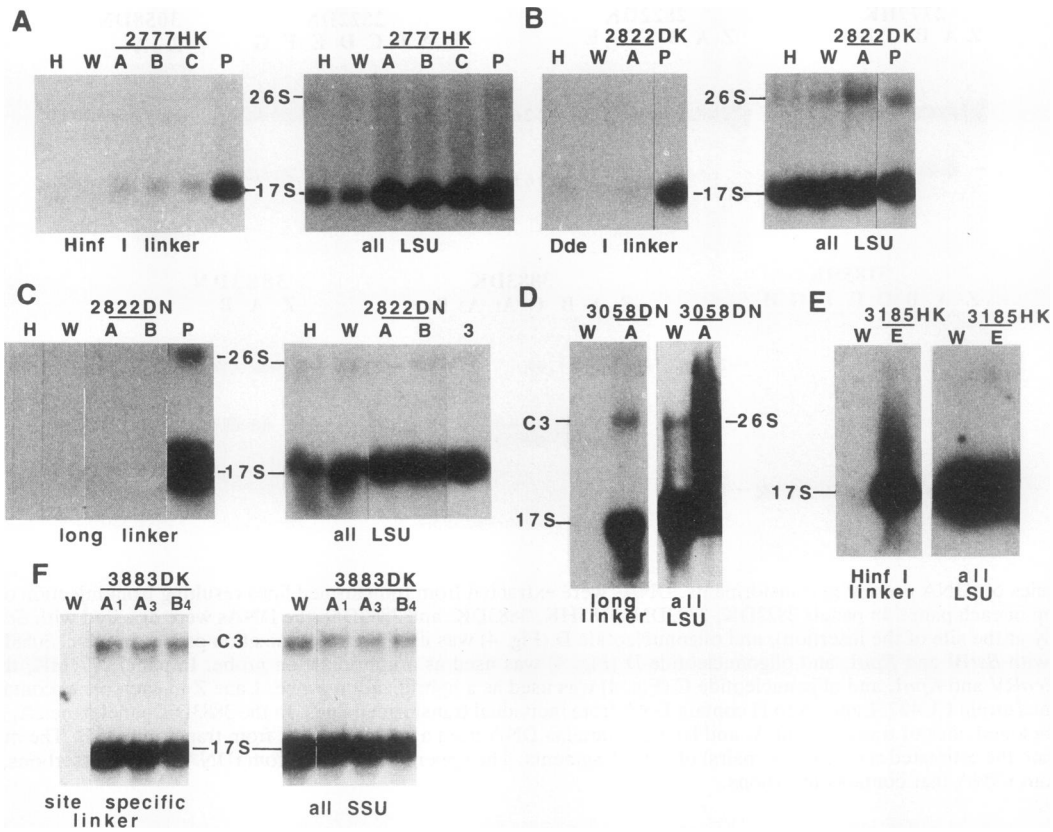

FIG. 6. Species of rRNA present in transformants. Each panel shows two autoradiographs of a single Northern blot hybridized with an oligonucleotide that specifically detects the insertions (left autoradiograph) and an oligonucleotide that detects either all SSU or all large subunit (LSU) rRNAs (right autoradiograph), as indicated beneath each panel. The sequences of these oligonucleotides are 5'ACAAGC CAGTTATCCCTGTG3' (all large subunits), 5'ACCCAACTACGAGCTTTTTAACT3' (all SSU), 5'GGCCGCTTTAAAGGTTACCCCGGG GGGCCCGTCGACGC3' (long linker), 5'GANTCGGTACCGANTC3' (*Hin*fI linker), 5'CTNAGGGTACCCTNAG3' (*Dde*I linker), and 5'C CCTCTAAGGGTACCCTA3' (site-specific linker). Lanes H contain host type total RNA from one of the parental strains, CU427. Lanes W contain wild-type RNA from a 947-400 transformant. Lanes labeled with a construct name over a letter (A to E) or a letter plus a subscript (A_1, A_3, B_4) contain RNAs from individual transformant lines or subclones thereof, respectively. Individual transformant lines are designated below with a dash and a letter following the name of the construct (for example, 3058DN-A). Lanes P are positive controls which contain RNAs from a variety of transformant lines: panel A, 5785HK-K; panel B, 8498DK-A; panel C, 7002HN-B. The mature large subunit rRNA comprises two fragments of approximately equal size that comigrate with the mature SSU rRNA at 17S. Precursor species for the large subunit (labeled 26S) and SSU (labeled C3) rRNAs migrate at about 26S (18). The RNAs from transformants 5785HK-K, 8498DK-A, and 7002HN-B are used as positive controls here, and these transformants will be discussed in detail in another publication (39).

transformants could be pm-r when they apparently contain little or no mature rRNA bearing the insertion. One possible explanation is that these transformants contain rRNA molecules that contain the *Pmr* mutation but not the insertion. Such rRNAs could be encoded by rDNAs that had recombined between the *Pmr* mutation and the site of the insertion. Such molecules could be detected by cutting total transformant DNA with *Kpn*I (which has no sites in the wild-type rDNA but cleaves at the insertions) and using an oligonucleotide homologous to the *Pmr* mutant sequence as a hybridization probe for a Southern blot. Under stringent hybridization and washing conditions, one can detect the 1-bp sequence difference between the Pmr^+ and *Pmr* mutant sequences (1). If no recombination has occurred, the *Pmr* oligonucleotide should hybridize only to the 7.7-kb band (Fig. 9). If recombination has occurred, hybridization to uncut rDNAs or to rDNAs cut on only one side should also be detected.

The results of this experiment (Fig. 9) show that rDNA molecules that have recombined between the *Pmr* mutation and the site of the insertion are abundant in all five 2822DK transformants and in two of four 2777HK transformants. The presence of these recombinant rDNA molecules offers an explanation for the pm-r phenotype of these transformants. The remaining two 2777HK transformants contained low levels of recombinant rDNAs. Interestingly, these two transformants also grew more slowly in paromomycin (6-h doubling time compared with a 4-h doubling time). Our unpublished data suggest that cells containing about 10% *Pmr* rRNA can be pm-r. If so, it is possible that the low levels of recombinant rDNA molecules seen in two 2777HK transformants (A and D) are sufficient to produce enough rRNA bearing the *Pmr* mutation (and not the inserted sequence) to confer the pm-r phenotype.

DISCUSSION

Although much information concerning the structure and function of the SSU rRNA has been accumulated, a complete picture of the role of the SSU rRNA in translation is

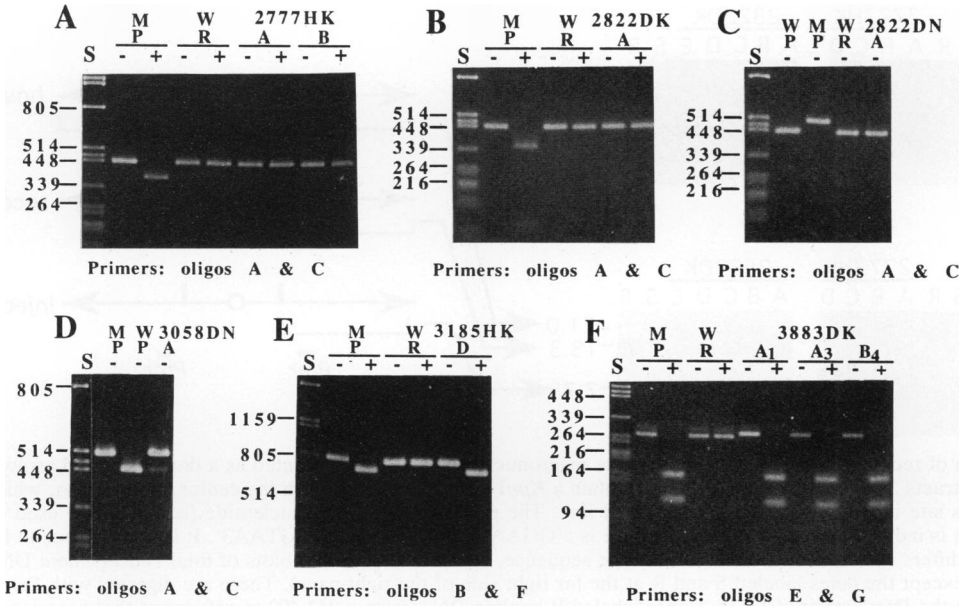

FIG. 7. Detection of transformant RNA by PCR. Reverse transcription followed by PCR was performed as described in Materials and Methods to simultaneously detect the presence of host and injected type rRNA. In all panels, lane S contains lambda DNA digested with *Pst*I. The oligonucleotides (Fig. 4) used as PCR primers are indicated beneath each panel. + or − above each lane indicates that the sample has (+) or has not (−) been digested with *Kpn*I, which cleaves at the insertion but not elsewhere in the rDNA. Lanes labeled with a construct name above a letter (A to D) or a letter with a subscript (A_1, A_3, B_4) contain PCR products derived from RNA of individual transformant lines or subclones thereof, respectively. Lanes labeled with an M above a P contain PCR products derived from bacterial plasmid DNA of the mutant construct used to produce the *Tetrahymena* transformants, whose RNA is examined in that panel. Lanes labeled with a W above an R contain PCR products derived from RNA of *Tetrahymena* transformants resulting from injection of a wild-type construct (947-400). Lanes labeled with a W above a P contain PCR products derived from 947-400 bacterial plasmid DNA.

lacking. In this study, we directly test the validity of some ideas about ribosome structure and function developed from in vitro work and sequence comparison by analyzing the phenotypic effects of small insertions at a number of sites in the *Tetrahymena* SSU rRNA. The mutation sites studied fall within regions that range from being quite variable to universally conserved in sequence. This work addresses the validity of the proposed tertiary structure models for the SSU rRNA (3, 24, 27, 38) in the sense that insertions that do not affect rRNA function would be expected to lie in areas where an insertion could be accommodated without disturbing the tertiary structure of the rRNA, which is presumably essential for ribosome function.

Some evidence exists concerning the effects of mutations in variable regions. In yeast cells, a variety of alterations within variable regions have been made in a system where the altered rDNA is carried on a plasmid that is present in only a few copies per cell, while the host cell rDNA is present in 100 to 200 copies. The altered rDNA is judged as functional or nonfunctional on the basis of whether or not the resulting rRNA shows a normal distribution into polysomes. Insertions have been placed into two variable re-

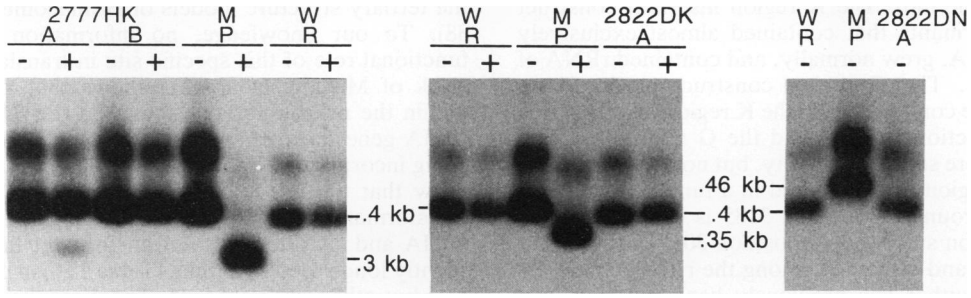

FIG. 8. Detection of precursor rRNA species. Reverse transcription followed by PCR was performed as described in Materials and Methods, except that 2.5 μg (rather than 1 μg) of total rRNA was used for each reaction. Primers for PCR were oligomers H and J (Fig. 4). Subsequent to electrophoresis, Southern blotting was performed, and the resulting blot was hybridized with oligomer A (Fig. 4) labeled on its 5′ end with [^{32}P]phosphate. The + and − signs above each lane indicate whether a sample has (+) or has not (−) been digested with *Kpn*I. Lanes labeled with a construct name above a letter (A or B) contain PCR products derived from total RNA of individual transformant lines. Lanes labeled with an M above a P contain PCR products derived from the bacterial plasmid DNA that was used to produce the transformants whose RNA is examined in the same panel. Lanes labeled with a W above an R contain PCR products derived from RNA from a cell line transformed with 947-400, a wild-type construct. The weaker band above the 0.4-kb band is presumably a PCR artifact, which is often detected in this sort of experiment.

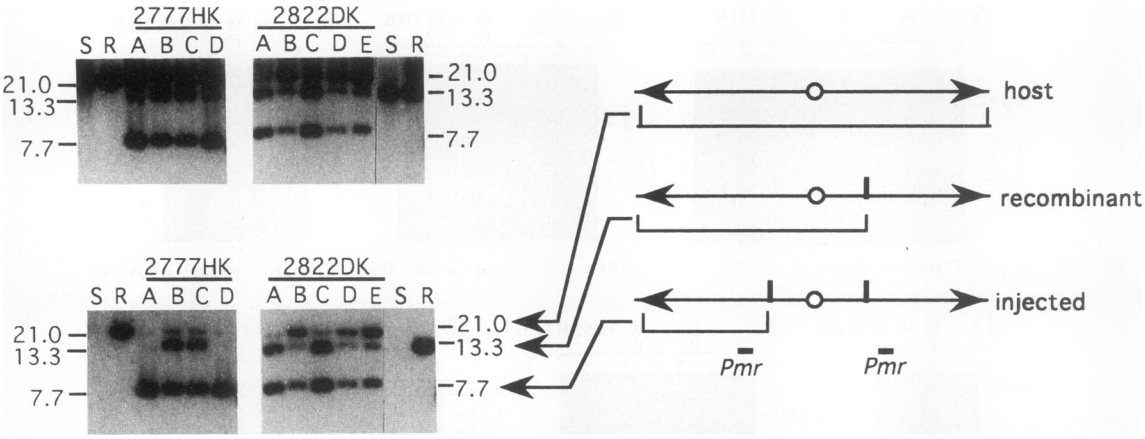

FIG. 9. Detection of recombinant rDNAs. At right, the macronuclear rDNA is represented as a double-headed arrow. Its total length is 21 kb. The two constructs 2777HK and 2822DK each contain a *Kpn*I site about 2.8 kb from the center of the rDNA, which is not present in the host rDNA. This site is indicated by a short vertical line. The position of the oligonucleotide (labeled *Pmr*) used to hybridize to the Southern blots at left is indicated below, and its sequence is 5′GGAACAACAAAAAGTCGTAA3′. It is homologous to the sequence of the *Pmr* mutant, which differs by one base from the wild-type sequence. At left are Southern blots of total *Tetrahymena* DNAs. All DNAs are digested with *Kpn*I except the lanes labeled S and R at the far right side of the right panel. These are digested with *Bam*HI. Lanes labeled S contain DNA from the Pmr^+ strain, CU427. Lanes labeled R contain DNA from a 947-400 transformant that contains *Pmr* rDNA. Lanes labeled with a construct name above letters (A to E) contain DNA from individual transformant lines. The blots shown in the upper two panels were washed in 2× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA [pH 7.7]) (14) at room temperature, and the blots shown in the lower two panels were stringently washed in 0.1× SSPE for 40 min at 37°C.

gions, V2 and V7, (according to the nomenclature of Neefs et al. [25]) in the SSU rRNA gene (21) and into variable region A (according to the nomenclature of Gutell and Fox [7]) in the large subunit rRNA gene (23) of *Saccharomyces cerevisiae*. SSU rRNA containing the V2 insertion could not be detected in Northern blots, although the altered rDNA was present in transformant cells. Musters et al. (21) presumed that this rRNA is rapidly degraded. The other two insertion constructs produced rRNAs that could not be distinguished from wild-type rRNA in their distribution into polysomes. Musters et al. have also deleted most of the *S. cerevisiae* variable region E (7) in the large subunit rRNA gene and replaced the missing bases with a similarly placed variable region from mouse rDNA (22). Both of these constructs produce rRNAs that show a normal polysome distribution. In the *Tetrahymena* system in which the altered rDNA can completely replace the somatic rDNA, insertions were placed into variable regions G and K (7) in the large subunit rRNA gene (40). The K region insertion construct produced transformants that contained almost exclusively injected type rDNA, grew normally, and contained rRNA of the injected type. The G region construct produced no transformants. We concluded that the K region insertion was tolerated in a functional rRNA and the G region insertion was not. It therefore seems that many, but not all, alterations within variable regions are tolerated in a functional rRNA.

The region surrounding position 3185 is extremely variable, and the region surrounding position 3058 is somewhat variable in length and sequence among the rRNAs of different organisms. Neither has previously been mutated in a functional study. In both 3058DN and 3185HK, mutated rDNA replaces host rDNA almost completely in the transformants, and the mutation is also present in almost all copies of the rRNA. Transformants grow as well as cells containing rDNA bearing the *Pmr* mutation that is otherwise wild type. We conclude that the insertions that we have introduced into these two variable regions can be accommodated in a functional ribosome.

We propose that insertions (such as those at positions 3058 and 3185) that do not affect ribosome function must lie in positions in which they can be accommodated without disturbing essential features of the tertiary structure. For example, a likely position for these sites would be on the surface of the SSU but not on the side that interfaces with the large subunit. These constraints are satisfied by the Noller model (38) for both sites 3058 and 3185, by the Lake and Brimacombe models (3, 27) for site 3058, and by the model of Nagano and Harel (24) for neither.

Site 3883 lies within sequences that are highly conserved among eucaryotes and are part of a long, double-stranded stem with bulges seen in all SSU rRNAs except those from some mitochondria. Some sequence conservation at this site is also seen within other kingdoms. Approximately 5 to 10 bp beyond the 3883 site in this stem structure, the length and sequence of rRNAs from different organisms become variable. The 3883 site lies in an area proposed to be crowded in the tertiary structure models of Brimacombe and Noller (3, 38). To our knowledge, no information concerning the functional role of this specific site in translation exists. The work of Musters et al. (21) shows that a small insertion within the nearby variable region in the *S. cerevisiae* SSU rRNA gene does not prevent the altered SSU rRNA from being incorporated into polysomes in vivo. In this study, we show that the injected type rDNA and rRNA in 3883DK transformants almost completely replace the host type rDNA and rRNA, and the transformant lines grow with a slightly longer doubling time (Table 1) than cell lines that are *Pmr* but otherwise wild type. These results suggest that this alteration may have a slight deleterious effect on rRNA function. A longer insertion at this site (as in 3883DN) appears to seriously compromise rRNA function, since transformants resulting from this construct die when maintained under selection. A possible interpretation of these results is that the specific sequence at the 3883 site is not important for rRNA function but that a sufficiently large insertion at this site disturbs rRNA structure enough to

abolish function. Our data suggest either that there is sufficient space in this area to accommodate the small inserted sequence or that a small distortion in the tertiary structure in this area does not seriously affect ribosome function.

In some cases (2777HK, 2822DK, and 2822DN transformants), the host rDNA is partially replaced by injected rDNA, and transformant lines containing only injected rDNA cannot be obtained. Surprisingly, rRNA bearing the alteration is undetectable in either Northern blots (Fig. 6) or in the PCR assay with primers within the mature SSU rRNA coding region (Figure 7), in spite of the fact that rDNA bearing the alteration comprises a substantial proportion of the rDNA in these cells. In two 2777HK transformants, we detected minor amounts of precursor species bearing the inserted sequence (Fig. 8 and Results). These data indicate that the 2777HK rDNA can be transcribed. Although we suspect so, we cannot be certain that it is transcribed at a normal rate. We suggest that some preferential degradation of injected type rRNA has occurred both prior to and after the precursor stage rRNA that we have detected in these transformants. Since we have failed to detect any precursor rRNAs in transformant lines 2822DK-A and 2822DN-A, we estimate that injected type rRNA precursor molecules are at least 20-fold less abundant than host type rRNA precursor molecules. This estimate is based on previous experiments in which rDNA species comprising 1% of the total cannot be detected and species comprising 5% of the total can be detected by PCR in our hands (data not shown). We conclude that the rRNA bearing either of these three insertions is inefficiently transcribed and/or rapidly degraded. Since both of these insertion sites are distant from sequences thought to be involved in the regulation of rRNA transcription, it is most likely that transcripts containing the insertions are unstable.

Insertions at two positions (2919 and 3694) resulted in a failure to transform. The sequences surrounding positions 2919 and 3694 are similar in that both are very highly conserved and are within single-stranded regions of the rRNA secondary structure. The high degree of conservation suggests that these sequences may play a sequence-specific role in translation. A base analogous to site 3697 in the *E. coli* rRNA (the G at position 966) has been shown to be involved in tRNA binding to the P site (17). To our knowledge, the region surrounding site 2919 has not been associated with any ribosomal function. Since we obtained no transformants with either 2919DK or 3694HK, our experiments cannot directly address the question of whether ribosomes containing rRNAs bearing these alterations are functional. We feel that a failure to transform implies that these altered rDNAs are nonfunctional: this could mean failure to serve as an rRNA template, instability of the rRNA, failure of the rRNA to be processed or assembled into ribosomes, or failure of the ribosomes to function.

What is the difference between constructs like 2777HK and 2822DK, which produce transformants that contain undetectable amounts of mature rRNA bearing the inserted sequence, and constructs like 2919DK and 3694HK, which produce no transformants? pm-r transformants resulting from 2777HK and 2822DK are apparently able to survive because they contain rDNA molecules that have recombined between the site of the insertion and the site of the *Pmr* mutation to produce rDNAs bearing the *Pmr* mutation but not the insertion. If such recombination could occur in the cases of 2919DK and 3694HK, presumably transformants would also be obtained, unless these constructs exert a dominant lethal effect. We have observed recombination of rDNAs during vegetative growth to be quite frequent, but we do not know whether recombination is sufficiently frequent in the intervals between 2919 and the *Pmr* site at position 4242 or between 3694 and 4242 to promote transformation in cases where it would otherwise not occur. We conclude either that recombination is not sufficiently frequent to promote transformation or that these constructs exert dominant lethal effects.

In summary, we have studied the phenotypes of cells bearing insertions at seven different sites within the SSU rRNA gene of *T. thermophila*. Constructs bearing insertions at two sites (2919 and 3694) within universally conserved sequences failed to transform. We conclude that bases within these regions fulfill essential roles in rRNA maintenance, processing, assembly, or function. This is consistent with what would be expected for universally conserved sequences. Insertions at two other sites (2777 and 2822) cause the resulting rRNAs to be either inefficiently transcribed or rapidly degraded. Since these alterations are distant from sequences thought to be involved in the regulation of transcription, it is more likely that these rRNAs are rapidly degraded. In the case of 2777HK, we have shown that rRNA precursor molecules containing the insertion are present at subnormal levels in the transformants. Therefore, this altered rDNA is transcribed, although the rate of transcription may or may not be normal. Constructs bearing insertions at three other sites (3058, 3185, and 3883) behaved essentially like unaltered constructs. We propose that the sequences surrounding these sites play no essential roles in ribosome structure or function and are located in positions that can accommodate an insertion without altering the overall tertiary structure of the ribosome. This is not surprising for sites 3058 and 3185, since they lie within variable regions, but is unexpected for site 3883, since it lies in a well-conserved part of the rRNA secondary structure which is proposed to lie in a crowded portion of the tertiary structure. The slight growth defect seen in transformants containing only 3883DK rRNA may be due to a slight functional defect in this rRNA.

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