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# A Mutation in the Large Subunit Ribosomal RNA Gene of Tetrahymena Confers Anisomycin Resistance and Cold Sensitivity

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ABSTRACT

Anisomycin, an antibiotic that specifically inhibits the peptidyl transfer function of eukaryotic ribosomes, has been used to select resistant mutants in *Tetrahymena thermophila*. A mutation conferring anisomycin resistance (an-r) has been localized to a 1.2-kb fragment of the large subunit ribosomal RNA (rRNA) gene by transformation via microinjection. A single base pair change was detected within this region. Nine independently isolated an-r mutants had the same base pair change. *T. thermophila* strains that are homozygous for this mutation are cold sensitive, unable to mate and grossly abnormal in cell morphology.

ALTHOUGH information on the physical structure of the rRNAs is abundant, functional information, particularly in eukaryotic versions of the rRNAs, is scarce. Classical genetic studies have been hampered by the multiple copies of the rRNA genes that exist in most genomes. Thus, most studies have been restricted to the mitochondria of higher cells. *Tetrahymena thermophila,* a single-celled eukaryote, is ideally suited to genetic studies of the rRNA genes since the organism is amenable to genetic manipulation (ORIAS and BRUNS 1976) and is the only eukaryote known to possess only one copy of the rRNA genes (YAO and GALL 1977). Moreover, *T. thermophila* can be transformed by microinjection of the rRNA genes (rDNA), which provides a simple way to map mutations within the rDNA (TONDRAVI and YAO 1986; YAO and YAO 1989).

*T. thermophila,* like many other ciliates, possesses two types of nuclei per cell: a micronucleus, or germline nucleus, and a macronucleus, or somatic nucleus, in which the vast majority of transcription takes place. The micronucleus is diploid, possessing five pairs of chromosomes. There is one copy of the rRNA genes on the left arm of chromosome *2* (BRUNS *et al.* 1985; BRUNS 1990). In the macronucleus (which is derived from a nucleus identical to the micronucleus during conjugation) the chromosomes are fragmented in a specific manner into hundreds of pieces with an average size of 600 kb. Most of these, with the exception of the rDNA, are present in about 45 copies per cell (reviewed in YAO 1989). Two copies of the rRNA genes arranged in a giant palindrome reside on a 21-kb DNA molecule present at about 9000 copies per cell (reviewed in YAO 1986).

During conjugation, cells having different mating types form pairs, and the micronucleus of each partner produces four meiotic products. Three of these are resorbed by the cell, and one becomes the gametic nucleus, which replicates itself. The partners then exchange nuclei, each donating one gametic nucleus to the other. When this process is complete, each partner possesses two haploid gametic nuclei, one derived from the genome of each parent. These gametic nuclei fuse to form a new diploid zygotic nucleus. The old macronuclei of both partners are resorbed by the cells, and new macronuclei develop from daughter nuclei of the new zygotic nucleus. The partners then separate and resume vegetative growth. The process of conjugation and its consequences is reviewed in BRUNS (1986) and ORIAS (1986).

A few regions of the large subunit rRNA, which, together, comprise a very small fraction of its entire length, have been implicated in specific functions of the ribosome. One of these is a single stranded ring of bases in "domain V" (NOLLER 1984) that has been implicated in the peptidyl transfer function of the ribosome, which will be referred to as the peptidyl transfer center (see Figure 1). Many mutations conferring various drug resistances have been found in the peptidyl transfer center. These include mutations conferring resistance to drugs that specifically inhibit peptidyl transfer, such as chloramphenicol and anisomycin, as well as to drugs that inhibit translocation, such as erythromycin (HUMMEL and BOCK 1987; NOLLER 1984; GALE *et al.* 1981). In addition, binding of chloramphenicol and erythromycin to ribosomes *in vitro* protects bases in the peptidyl transfer center from chemical attack (MOAZED and NOLLER 1987), suggesting that these bases may be a physical part of the binding sites of these drugs. The amino acyl end of a ribosome-bound tRNA would be expected to be physically close to bases participating in the peptidyl

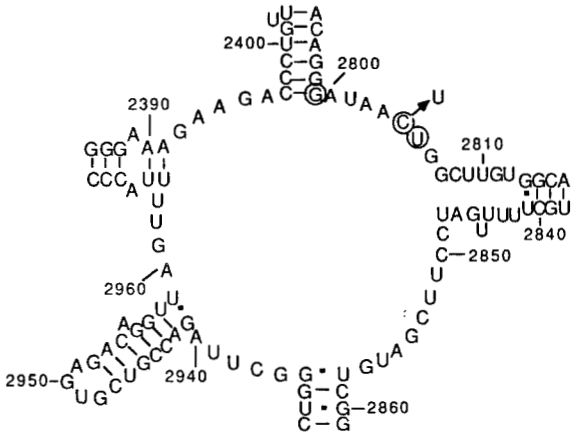


FIGURE 1.—Sequence of the *Anr* mutation. A portion of the *T. thermophila* large subunit rRNA, which was folded on the basis of phylogenetic comparison with large subunit rRNAs from other organisms (R. GUTELL, personal communication), is shown. This single stranded ring of bases has been implicated in the peptidyl transfer function of the ribosome in a variety of organisms. The three circled bases are the sites of mutations conferring an-r in Halobacterium. The circled C with an arrow pointing to a U shows the base change found in the *T. thermophila* an-r mutants isolated in this study. The numbers refer to the position of the bases from the beginning of the 26S rRNA. Corresponding numbers starting from the center of the palindromic macronuclear rDNA (H. NIELSEN, personal communication) are as follows: 26S rRNA positions 2400, 2800, 2850 and 2940 correspond to rDNA positions 7562, 7962, 8012 and 8102.

transfer reaction. Several bases in the peptidyl transfer center have been positioned close to the amino acyl end of a photo-reactive derivative of yeast Phe-tRNA by cross-linking (STEINER, KUECHLER and BARTA 1988). Taken together, this evidence strongly suggests that bases in the peptidyl transfer center of the large subunit rRNA actually do participate in the peptidyl transfer reaction.

Anisomycin is a drug that specifically inhibits the peptidyl transfer reaction in eukaryotic cells and some archaebacteria (GALE *et al.* 1981). Mutations conferring an-r have been isolated in Saccharomyces (GRANT, SCHINDLER and DAVIES 1976; JIMENEZ and VAZQUEZ 1975), Methanobacterium (HUMMEL and BOCK 1985), Chlamydomonas (JAMES and LEFEBVRE 1989) and Halobacterium (HUMMEL and BOCK 1987). The an-r phenotype in mutants of Saccharomyces has been shown to be the result of an altered ribosomal protein L3 (FRIED and WARNER 1981), while an an-r mutant in Chlamydomonas is most likely altered in a gene encoding a product that affects drug uptake or export (JAMES and LEFEBVRE 1989). An-r mutations have been correlated with sequence alterations in the large subunit rRNA gene in Halobacterium. The sequence changes detected were in the peptidyl transfer center. Halobacterium, like *T. thermophila,* has only one copy of the rDNA, and thus, it was possible to isolate mutations in their rDNA. In the present study, we isolate an-r *T. thermophila* mutants and show that the sequence changes seen in their rDNA are identical to each other and to that of a class of an-r mutants isolated in Halobacterium. In addition, we show that strains homozygous for this mutation are cold sensitive for growth and have an aberrant cell morphology. To our knowledge, this is the first time a drug-resistance mutation has been isolated in the large subunit rRNA gene of a eukaryote.

## MATERIALS AND METHODS

**Strains, media and cell culture:** Cells were grown in suPP media (GOROVSKY 1970), which consists of 2% proteose peptone, 0.2% dextrose, 0.1% yeast extract and 0.003% sequestrine. The media is initially prepared at ten times this concentration and centrifuged at 4900 × *g* for 20 min. It is then stored at −20° and thawed, diluted ten fold, and autoclaved prior to use. Genetic manipulations were performed as described (ORIAS and BRUNS 1976). All strains are described in Table 1.

**Isolation of mutants:** Two crosses were done in order to obtain an-r mutants carrying B or C3 type rDNA. Many sequence differences exist between C3 type rDNA (derived from a C3 inbred strain) and B type rDNA (derived from a B inbred strain). One or more of these sequence differences confers a replication advantage on C3 type rDNA such that a newly formed C3/B heterozygote has almost exclusively C3 type rDNA in its macronucleus within about twenty cell doublings subsequent to the formation of the macronucleus (LARSON *et al.* 1986). A single an-r isolate of each rDNA type was obtained by mutagenizing either *CU428* (B type) or *C3–368* (C3 type) with *N*-methyl-*N'*-nitro-nitrosoguanidine (ORIAS and BRUNS 1976) and immediately starving for about 17 hr (in 10 mM Tris, pH 7.4) preparatory to mating to strain *CU427,* which had been starved a similar amount of time. The mating cells (in 10 mM Tris, pH 7.4) were fed with one volume of suPP media 24 hr after mixing. After about two cell doublings (approximately eight hours), cycloheximide (25 μg/ml) was added. After 2 days in cycloheximide, cells were washed into suPP media containing anisomycin (100 μg/ml) with no significant dilution, and after three to five days single an-r cells were isolated from these cultures. Initially, a single isolate of each rDNA type was sought and obtained. Subsequently, multiple independent isolates of the B type were sought by distributing a mating mixture of $2.4 \times 10^5$ mutagenized cells (*CU428*) plus $2.4 \times 10^5$ unmutagenized cells (CU427) into ten flasks at the time of feeding with suPP media. Seven out of these ten flasks produced at least one an-r cell. Taking into consideration the pairing efficiency, the pair viability and the percentage of pairs that produced true (cycloheximide-resistant) progeny, an-r mutants were obtained at a rate of at least $1.8 \times 10^{-4}$.

**Cloning, sequencing and oligonucleotide hybridizations:** Standard techniques were used to clone a portion of the an-r rDNA into a bacterial vector (MANIATIS, FRITSCH and SAMBROOK 1982). The region in question was sequenced using the double stranded sequencing protocol included with the "Sequenase" kit (U.S. Biochemical). Oligonucleotide hybridizations to distinguish small sequence changes were performed as previously described (AUSTERBERRY and YAO 1987).

**Strain construction:** The strains *A*III* and *B*VI* (known as star strains) are defective in their micronuclei. A mating between a normal strain and a star strain is different from a mating between two normal strains and is referred to as a

genomic exclusion mating (ALLEN 1967). The partners pair normally, and the micronucleus of the normal partner undergoes meiosis producing four haploid gametic nuclei. Three of these are resorbed by the cell, and one is replicated. One replicate is donated to the star partner, and both partners replicate their haploid nuclei to form a diploid nucleus. The partners separate, and they are referred to as round one exconjugants. Both exconjugants have identical micronuclei which are derived exclusively from the genome of the normal partner and are homozygous for their entire genome but retain the parental macronuclei.

We designate *Anr* to be a 26S rRNA gene that bears a specific mutation (described in the present study) that confers an-r. The wild type allele is designated $Anr^+$.

*FH101* was created by mating *FH106* (a product of the an-r mutant selection scheme described in a previous section) to *A*III* and isolating round one exconjugant pairs. These round one exconjugants were tested by crosses to *CU428* for the presence of the *Anr* and *ChxA* alleles in their micronuclei. The partners of one pair, both of which were homozygous for the *Anr* and *ChxA* alleles, were crossed to each other to produce *FH101*. It should be noted that the *FH106* strain used in this construction was not expressing cycloheximide resistance (cy-r) in its macronucleus, even though it was heterozygous for *ChxA* in its micronucleus. This was presumably due to phenotypic assortment (SONNEBORN 1974).

In addition, an-r progeny (which were B-*Anr*/B-$Anr^+$ in genotype), produced in the test crosses of the round one exconjugants just mentioned with *CU428,* were crossed to *A*III* to produce a second set of round one exconjugants. These were tested for the presence of the *Anr* allele in their micronuclei. One of these, which had the macronucleus of *A*III* and was B-*Anr*/B-*Anr, Mpr/Mpr, ChxA/ChxA* in its micronucleus, was *FH104*.

*FH103* was made by crossing *FH107* (a product of the previously described an-r mutant selection scheme) to *CU428* and selecting an-r, cy-s and mp-r progeny (which were *C3-Anr*/B-$Anr^+$, $Chx^+/Chx^+$, $Mpr/Mpr^+$ in genotype). The progeny were then crossed to *CU427,* and an-r, cy-r, mp-r progeny (which were C3-*Anr*/B-$Anr^+$, $Chx/Chx^+$, $Mpr/Mpr^+$ in genotype) were selected. These progeny were then mated to *B*VI,* and round one exconjugant pairs were isolated and tested for the presence of the *Anr* allele in their micronuclei. *FH103* was one of these round one exconjugants which possessed the macronucleus of *B*VI* and a micronucleus with the genotype C3-*Anr*/C3-*Anr, Mpr/Mpr,* $Chx^+/Chx^+$.

*FH105* is an an-r, cy-r, mp-r progeny of a cross between *FH103* and *FH104* with the micronuclear genotype C3-*Anr*/B-*Anr, Chx/Chx*$^+$, *Mpr/Mpr. FH102* was constructed by crossing *FH103* to *A*III* and producing round one exconjugants. Exconjugant partners were crossed, and progeny were selected with anisomycin. One of these an-r progeny was *FH102*.

## RESULTS

**Isolation of an-r mutants:** Two rDNA markers previously characterized in *T. thermophila* have been used in these studies. One of these, *Pmr,* confers resistance to paromomycin (pm-r) and is a single base change near the 3′ end of the 17S rRNA gene (BRUNS *et al.* 1985; SPANGLER and BLACKBURN 1985). Another, C3 type rDNA (derived from C3 inbred strains), confers replication dominance over B type rDNA (derived from B inbred strains; PAN *et al.* 1982; LARSON *et al.* 1986): that is, when rDNAs of each of these types are present in a newly formed macronucleus, the rDNA of the C3 type becomes the only rDNA in the macronucleus after about 20 rounds of cell doubling. C3 type rDNA contains many sequence differences from B type rDNA. However, the difference responsible for the replication dominance is probably within the replication origin region (LARSON *et al.* 1986; LOVLIE, HALLER and ORIAS 1988; YAEGER *et al.* 1989), about 0.6 kb from the palindromic center of the 21-kb palindromic macronuclear rDNA molecule (CECH and BREHM 1981).

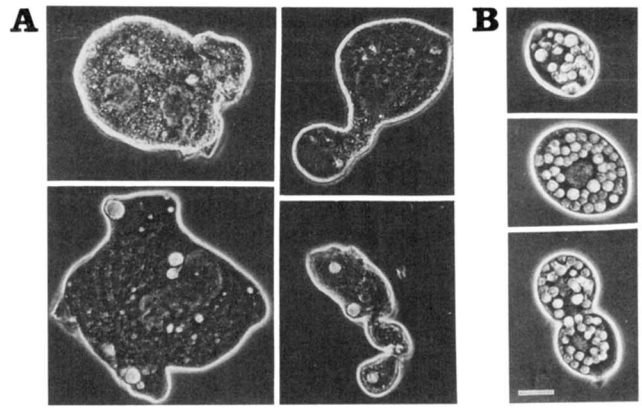


FIGURE 2.—Cell morphology of *Anr/Anr* homozygotes. Panel A shows four cells from strain *FH101* displaying the abnormal cell morphology characteristic of *Anr/Anr* homozygotes. Panel B shows three wild-type cells, one of which is about to divide. All cells were photographed using phase microscopy at a magnification of 320× and are shown at a magnification of 860×. The bar in panel B represents a distance of 10 μm.

An-r mutants were isolated using the selection procedure described in MATERIALS AND METHODS. Initially, two an-r mutants were obtained, one which contained exclusively B type rDNA in its macronucleus (*FH106*) and one which contained almost exclusively rDNA of the C3 type in its macronucleus (*FH107*). These mutations will be refered to as *Anr*. The wild type allele will be refered to as $Anr^+$. Subsequently, seven additional an-r mutant clones of independent origin were isolated from the same cross that generated *FH106* at a frequency of at least $1.8 \times 10^{-4}$. These mutants were presumably heterozygous for the mutation conferring an-r. Therefore, the mutation conferring an-r is dominant. However, the terms "dominant" and "recessive" are somewhat ambiguous in Tetrahymena since the macronucleus divides amitotically and, when heterozygous, is capable of producing individual macronuclei with widely varying proportions of the allele in question. But since these mutants were subjected to selection in anisomycin after relatively few cell doublings (*i.e.,* a limited opportunity to produce individuals with widely varying proportions of the *Anr* allele), it is likely that the *Anr* allele actually is dominant over the $Anr^+$ allele in

**TABLE 1**

**Strains**

| Strain | Micronucleus: Type of rDNA | Micronucleus: Other markers | Macronucleus: Type of rDNA | Macronucleus: Phenotype: cy-r | mp-r | an-r | pm-r | Mating type | Source |
|---|---|---|---|---|---|---|---|---|---|
| *CU427* | B/B | *ChxA/ChxA* | B | − | − | − | − | VI | P. BRUNS |
| *CU428* | B/B | *Mpr/Mpr* | B | − | − | − | − | VII | P. BRUNS |
| *FH101* | B-*Anr*/ B-*Anr* | *Mpr/Mpr*, *ChxA/ChxA* | B | + | + | + | − | −[a] | This study |
| *FH102* | C3-*Anr*/ C3-*Anr* | *Mrp/Mpr* | C3 | − | + | + | − | − | This study |
| *FH103* | C3-*Anr*/ C3-*Anr* | *Mpr/Mpr* | B | − | − | − | − | VI | This study |
| *FH104* | B-*Anr*/ B-*Anr* | *Mpr/Mpr*, *ChxA/ChxA* | B | − | − | − | − | III | This study |
| *FH105* | B-*Anr*/ C3-*Anr* | *Mpr/Mpr*, *ChxA/Chx*$^+$ | C3 | + | + | + | − | − | This study |
| *C3-368* | C3/C3 | | C3 | − | − | − | − | V | P. BRUNS |
| *A*III* | * | * | B | − | − | − | − | III | P. BRUNS |
| *B*VI* | * | * | B | − | − | − | − | VI | P. BRUNS |
| *FH106* | B-*Anr*/ B-*Anr*$^+$ | *Mpr/Mpr*$^+$, *ChxA/Chx*$^+$ | B | + | + | + | − | ND[b] | This study |
| *FH107* | C3-*Anr*/ B-*Anr*$^+$ | *ChxA/Chx*$^+$ | C3 | + | − | + | − | ND | This study |
| *SB255* | B/B | | B | − | − | − | − | IV | E. ORIAS |
| *SL062* | C3-*Pmr*/ B-*Pmr*$^+$ | | C3 | − | − | − | + | ND | This laboratory |

[a] A "−" means that the strain fails to form pairs with wild-type strains.
[b] ND means that the mating type was not determined.

cells containing equal numbers of these two alleles. It was noticed that eight out of eight isolates examined grew slowly and that a subset of the cells in cultures of these isolates had an unusual morphology similar to what was later observed in *Anr* homozygotes (Figure 2).

**Characterization of the phenotype of strains expressing only the *Anr* allele:** It was of interest to characterize the phenotype of a mutant that was expressing only the *Anr* allele in its macronucleus since *Anr/Anr*$^+$ heterozygotes grew slightly slower than wild type strains and had a subpopulation of cells with very aberrant morphology. The severity of both of these phenotypes varied from strain to strain in *Anr/Anr*$^+$ heterozygotes, presumably reflecting varying proportions of the *Anr* allele in the macronuclei. Since genetic background may affect phenotype, *Anr/Anr* homozygotes were constructed in three different genetic backgrounds (*FH101*, *FH102* and *FH105*; Table 1) by genomic exclusion mating as described in MATERIALS AND METHODS. These *Anr* homozygotes were characterized for growth at 37°, 30°, 23°, and 18° (Figure 3). All failed to grow at 18° and grew very slowly or not at all at 23°, although it was clear from microscopic examination that most of the cells in the culture were still alive after one week at these temperatures. Wild-type cells, however, grew at all temperatures tested.

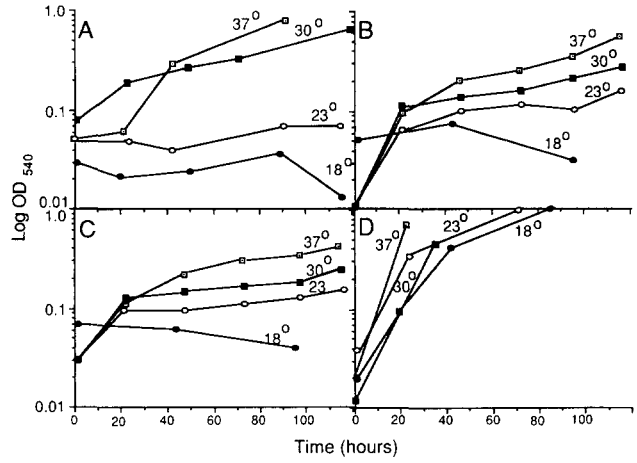


FIGURE 3.—Growth curves of *Anr/Anr* and wild-type strains. Growth was monitored by the optical density at 540 nm. An $OD_{540}$ of 0.2 corresponds to approximately $2 \times 10^5$ cells/ml. Cultures for growth measurements were started from cultures grown at 30° or 37° that were in log phase as judged visually or that had an $OD_{540}$ between 0.05 and 0.4. These were then diluted to an $OD_{540}$ of between 0.012 and 0.08 and placed at the temperature in question. Cells were grown without shaking in 50 ml flasks in suPP media in volumes ranging from 5 to 20 ml. Each graph represents a strain: A, *FH101*; B, *FH105*; C, *FH102*; and D, *CU427*. Symbols indicate different temperatures: open squares, 37°; filled squares, 30°; open circles, 23°; and filled circles, 18°.

A subset of these *Anr/Anr* mutant cells (including the vast majority of the cells at 18° or 23° and fewer cells at higher temperatures) had an aberrant mor-

phology (Figure 2). This abnormal morphology included such features as irregular shape, large size, disorganized arrangement of cilia, abnormal swimming behavior, multiple macronuclei and lack or scarcity of food vacuoles. In addition, three *Anr/Anr* homozygotes strains tested at 30° failed to form pairs with wild type strains.

The phenotypes other than an-r associated with *Anr/Anr* homozygous strains seem to be recessive, although, as previously mentioned, an assignment of dominance or recessivity is necessarily less precise in Tetrahymena than it is in a typical organism. In the case of mating proficiency, the result is clear: *Anr/Anr* homozygotes fail to mate while *Anr/Anr*$^+$ heterozygotes mate normally. Though abnormal cell morphology is observed in *Anr/Anr*$^+$ heterozygotes, the severity of the phenotype and the proportion of the cell population exhibiting the phenotype is far greater in *Anr/Anr* homozygotes. Although the growth characteristics of *Anr/Anr*$^+$ heterozygotes have not been well characterized, it is quite clear that *Anr/Anr*$^+$ heterozygotes grow far faster than *Anr/Anr* homozygotes at 30° and only slightly slower than a wild-type strain.

**Determination of the level of drug resistance in an-r and wild-type strains:** Two wild-type and two *Anr/Anr* strains were tested for their levels of resistance to anisomycin, paromomycin and cycloheximide (Table 2). Wild-type strains were viable in anisomycin at 5 μg/ml and dead after three days in 25 μg/ml of anisomycin, whereas the *Anr/Anr* strains were viable at up to 300 μg/ml of anisomycin. No differences in resistance to paromomycin or cycloheximide were detected other than the expected resistance of *FH105* to cycloheximide.

**Mapping and sequencing of the an-r mutation:** To localize the mutation to the rDNA, the 21-kb macronuclear rDNA molecule was isolated (DIN and ENGBERG 1979) from *FH107*, a strain resulting from the previously described mutagenesis which was heterozygous for the *Anr* allele and for rDNA type (C3-*Anr*/B-*Anr*$^+$). This strain had exclusively C3 type rDNA in its macronucleus, about half of which was *Anr* and half of which was *Anr*$^+$, presumably due to somatic recombination (data not shown). This rDNA was injected into the strain SB255, which contains exclusively B type rDNA in its macronucleus, using a previously established method (TONDRAVI and YAO 1986). It was expected that the injected rDNA should become the predominant species in transformed cells because of its replication advantage. Anisomycin resistant transformants were obtained at frequencies of 25%, 26%, and 30% of the cells injected in three different experiments, thus demonstrating that the mutation confering an-r in this mutant resides within the rDNA.

**TABLE 2**

**Strain resistance**

| Strain name[a] | Cycloheximide (μg/ml) | | | Paromomycin (μg/ml) | | | | Anisomycin (μg/ml) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 5 | 25 | 25 | 65 | 85 | 100 | 5 | 25 | 300 |
| *FH105* | +[b] | + | + | + | NT[c] | NT | − | + | + | + |
| *FH102* | + | − | − | + | + | − | − | + | + | + |
| *CU427* | + | − | − | + | − | − | − | + | − | − |
| *CU428* | + | − | − | + | + | − | − | + | − | − |

[a] See Table 1 for the genotypes and phenotypes of these strains.
[b] A "+" indicates that the cells were alive and growing, and a "−" indicates that the cells were dead.
[c] NT means not tested.

In order to further localize and to sequence this an-r mutation, a 1.2-kb *Sph*I-*Stu*I fragment derived from the rDNA used in the injection experiment was used to replace a similar fragment in the rDNA vector Tt947-01 (Figure 4; YAO and YAO 1989), producing Tt947-315. Tt947-01 includes a micronuclear copy of the rRNA genes with a pm-r mutation in the 17S rRNA gene. When injected into developing macronuclei of mating cells, Tt947-01 is properly processed to form pm-r macronuclear rDNA at frequencies between 0.5% and 10% among the cells injected (YAO and YAO 1989). Injection of Tt947-315 produced transformants resistant to both anisomycin and paromomycin at a frequency of 3.2% (9 out of 277 injected cells) in one experiment and 1% (1 out of 102 injected cells) in another experiment, thus demonstrating that the mutation confering an-r is within the 1.2-kb *Sph*I-*Stu*I fragment. These transformants grew slowly in anisomycin, and cells displaying aberrant morphology were apparent in cultures of these transformants.

The plasmid Tt947-214 was constructed by replacing the 6-kb *Mlu*I-*Stu*I fragment of Tt947-01 with a similar fragment derived from the macronuclear rDNA of *FH107* (Figure 4). About 200 bp of this plasmid, including the segment corresponding to that in which an-r mutations were found in Halobacterium (HUMMEL and BOCK 1987), was sequenced and compared with the wild type sequence of the same inbred line (*C3-368*). A single base pair change was detected (Figure 1). It was identical to that found in one class of an-r mutants isolated in Halobacterium.

Though we had unambiguously shown that the mutation conferring an-r mapped within a 1.2-kb *Sph*I-*Stu*I fragment, it was a formal possibility that this mutation was outside the 200-bp region sequenced. Therefore, the remaining eight an-r isolates were tested for the presence of an identical base change by oligonucleotide hybridization. The results indicate that all nine independent isolates have the same sequence change in their rDNA (Figure 5). It thus seems virtually certain that this base change is responsible for the an-r phenotype.

**Rescue of *Anr/Anr* homozygotes by microinjection**

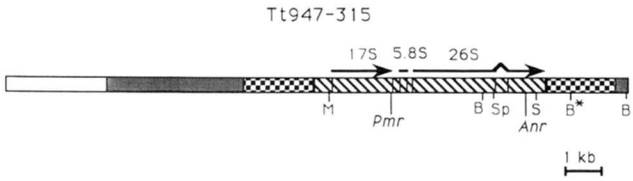


FIGURE 4.—Plasmid maps. This circular plasmid, Tt947–315, is shown as a linear that is cleaved at a polylinker sequence. Portions of the elongated rectangle depicting the plasmid DNA are filled differently to indicate the following DNA sequences: □, pUC19; ▩, micronuclear flanking sequences; ▨, non-transcribed spacer DNA; and ▧, transcribed rDNA. The horizontal arrows above indicate the coding regions for the 17S, 5.8S and 26S rRNAs. The intervening sequence is indicated by a bump in the horizontal arrow representing the 26S rRNA. Letters below the rectangle depicting the plasmid DNA indicate the following restriction sites: B, *Bam*HI; Sp, *Sph*I; M, *Mlu*I; and S, *Stu*I. The *Bam*HI site marked with an asterisk is present only in C3 type rDNA and not in B type rDNA. The positions of the mutations conferring drug resistance are indicated as *Anr* and *Pmr*. Tt947–01 is identical to Tt947–315 except that it is $Anr^+$. Tt947–214 is identical to Tt947–315 except that is is $Pmr^+$.

**of pm-r rDNA:** Though we had characterized *Anr/Anr* homozygotes in three different genetic backgrounds, it was still possible that the pleiotropic effects of the *Anr* mutation were, in fact, due to some genetic element in these strains other than the *Anr* mutation. We therefore planned a "marker rescue" experiment in which rDNA (derived from strain *SL062*) that was wild type at the *Anr* locus and mutant at the *Pmr* locus was injected into a strain homozygous for the *Anr* allele (*FH101*) and expressing only this allele in its macronucleus. Since the injected rDNA used in this experiment was C3 type and the host rDNA was B type, it was expected that injected rDNA should outreplicate the host rDNA and become the predominant species of rDNA in the pm-r transformants. If the pm-r transformants thus obtained failed to show the pleiotropic effects of the *Anr* mutation (for example, abnormal cell morphology, slow growth and failure to mate), this would indicate that an allele within the rDNA is necessary for these phenotypes.

One pm-r transformant of each of three independently isolated *Anr/Anr* homozygous strains was analyzed, and these grew with doubling times of 2.5–5.0 hr at 30° in the absence of paromomycin. Wild-type strains grew with doubling times of about 2.5 hr, and the untransformed *Anr/Anr* strains grew with doubling times of 16–17.5 hr under the same conditions (data not shown). In addition, the microscopic examination of the pm-r transformant cultures showed no cells with abnormal morphology. Four of these transformants were tested for their ability to mate. All paired normally. These transformants were not examined for growth at low temperature. We would conclude from these data that the phenotypes examined (growth rate at 30°, ability to mate and abnormal cell morphology) are specified mainly (if not exclusively) by a locus (or loci) on the rDNA.

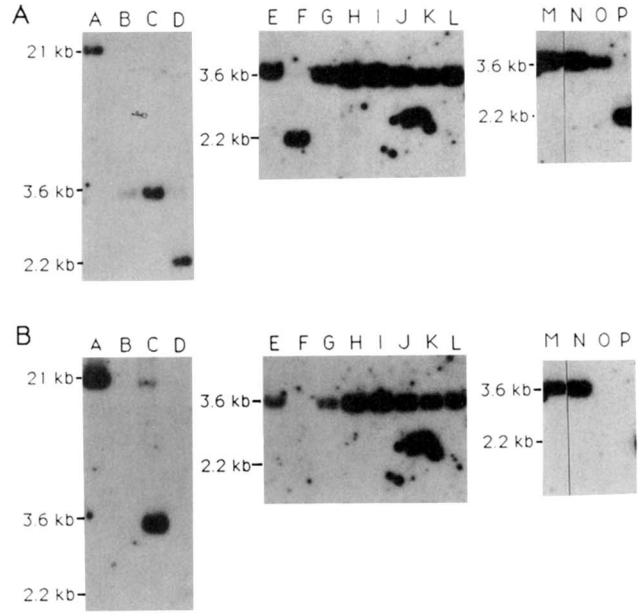


FIGURE 5.—Oligonucleotide hybridizations. Panels A and B show Southern blots of three gels that were hybridized with a 20 base oligonucleotide covering the site of and bearing the mutant sequence of the *Anr* mutation washed at low (panel A; 2 × SSC at room temperature) and high (panel B) stringency. All high stringency washes were done for 40 min at 37° in the following salt conditions: left blot, 0.0125 × SSC; middle blot, 0.00625 × SSC; and right blot, 0.1 × SSC. The sequence of the oligonucleotide is: ACAAGCCAATTATCCCTGTG. Lanes B, D, F, O and P contain total Tetrahymena DNA cut with *Bam*HI from strains bearing only an-s rDNA in their macronuclei of the B rDNA type (lanes B and O) or the C3 rDNA type (lanes D, F and P). Lanes C, E and N contain total Tetrahymena DNA cut with *Bam*HI from an *Anr/Anr* strain (*FH101*) bearing B type rDNA. Lanes A, G, H, I, J, K, L and M contain total Tetrahymena DNA from eight an-r mutant clones of independent origin derived from the same cross that generated *FH106* either uncut (lane A) or cut with *Bam*HI (all others).

## DISCUSSION

In this study we have shown that a single base change in the 26S rRNA gene confers resistance to anisomycin and, very likely, the other phenotypes observed in strains bearing this mutation. It is likely that the an-r phenotype of the *Anr* mutation is dominant in nature. By way of contrast, other phenotypes such as failure to mate, slow growth and abnormal cell morphology were observed only, or much more readily, in *Anr/Anr* homozygous strains and would thus seem to be recessive in nature. However, the terms "dominant" and "recessive" are somewhat ambiguous in Tetrahymena since the macronucleus divides amitotically and, when heterozygous, is capable of producing daughter nuclei with widely varying proportions of a given allele.

The mutation conferring an-r has been unambiguously localized to a 1.2-kb fragment of the rDNA by subcloning and transformation. Since not all the sequence within this fragment was determined, there remains the formal possibility that the actual mutation lies outside the sequenced region of the 1.2-kb frag-

ment. However, the fact that all nine an-r mutants that we have isolated in *T. thermophila* had the same base change in this region plus the fact that this base change is identical to one found in nine independent an-r mutant isolates in Halobacterium (HUMMEL and BOCK 1987) make this possibility seem very remote.

The phenotypes other than an-r observed in *Anr/Anr* homozygous strains are: inability to mate, abnormal cell morphology, cold sensitivity and slow growth at 30°. We believe these phenotypes result from the *Anr* mutation based on several lines of evidence. First, three of these phenotypes (inability to mate, abnormal cell morphology and slow growth at 30°) were mapped to the rDNA by transformation of *Anr/Anr* strains with rDNA bearing the *Pmr* and *Anr*$^+$ alleles. Second, three *Anr/Anr* strains with different genetic backgrounds express all four of these phenotypes, suggesting that these phenotypes are specified by a locus closely linked to the site confering an-r. Third, all independent isolates of *Anr/Anr*$^+$ strains examined (eight out of eight) grew slowly and displayed aberrant cell morphology in a subset of the cells. Fourth, all an-r transformants generated by microinjection of Tt947–315 (Figure 4), in which only a 1.2-kb *Sph*I-*Stu*I fragment of the rDNA had its origin in an an-r strain, grew slowly and displayed aberrant cell morphology. Taken together, this evidence convincingly argues that the phenotypes other than an-r displayed by *Anr/Anr* homozygotes are due to the presence of the *Anr* mutation.

Three classes of mutations conferring varying levels of an-r have been isolated in Halobacterium, an organism which, like Tetrahymena, possesses one copy of the rRNA genes. The sequence changes were determined by making informed guesses about where the mutation should be without the benefit of genetic mapping. This portion of the rDNA was then sequenced in multiple isolates (HUMMEL and BOCK 1987). One of these classes of mutations contained nine independent isolates which all bore an identical sequence change, which is also identical to the sequence change detected in the nine independent an-r mutants that we have isolated. Our finding thus lends additional support to the conclusion that the base change detected in Halobacterium is responsible for the an-r phenotype.

The area in which the *Anr* mutation occurs has been previously implicated in the peptidyl transfer function of the large subunit of the ribosome (NOLLER 1984). The mutation (a C to T transition) occurs at a site universally conserved in all sequenced large subunit rRNAs (GUTELL and FOX 1988). A C to A transversion at this same site confers resistance to chloramphenicol in human mitochondria (BLANC, ADAMS and WALLACE 1981). To our knowledge, this an-r mutation in *T. thermophila* is the first drug resistance mutation in a eukaryotic (nuclear) large subunit rRNA gene. Its existence demonstrates that this area also plays a vital role in peptidyl transfer in eukaryotes. The fact that the an-r mutation in *T. thermophila* and one of the an-r mutations in Halobacterium bear an identical base change at a absolutely invariant base lends confirmation to the widely held belief that structure-function relationships in the ribosome are universal.

*T. thermophila* mutants that are homozygous for the *Anr* mutation display several phenotypes besides an-r that appear to be independent of genetic background (slow growth, cold sensitivity, inability to mate and aberrant cell morphology). Since the base in question is invariant in all sequenced large subunit rRNAs, it would not be surprising that a change in this base could have profound effects on ribosome function. The phenotypes mentioned above could then be caused by defects in (or shortages of) gene products required for these functions. The cold sensitive phenotype could be due to defects in ribosome function or assembly at low temperature caused by the *Anr* mutation. It could be speculated that cold sensitivity is a consequence of an excessive tightening of a molecular interaction involving the large subunit rRNA which normally occurs, but must be transiently loosened or broken, during the translation cycle or ribosome assembly. At low temperatures, this interaction may become so stable that it prevents the normal progression of molecular events. In relation to this, it is of interest to note that a mutation in the peptidyl transfer center of the large subunit rRNA gene that confers erythromycin resistance in yeast mitochondria also confers cold sensitivity, probably as a result of incomplete assembly of the large subunit at low temperatures (CUI and MASON 1989). Assembly of either ribosomal subunit of *Escherichia coli* has long been known to be a process that cannot occur at low temperature (NOMURA and HELD 1974; DOHME and NIERHAUS 1976). It is possible that a cold-sensitive phenotype was not observed in the case of the Halobacterium an-r mutant because of the unusual growth conditions (which include 20% NaCl) of these organisms which would, presumably, have profound effects on molecular interactions within the cell. Aberrant cell morphology was also not noted in connection with the an-r mutants in Halobacterium, but perhaps such a phenotype would be more apparent in a larger and more complex organism such as *T. thermophila*. Alternatively, it is possible that the an-r mutants isolated in Halobacterium contained a suppressor mutation mapping outside of the sequenced region (either within or outside of the rDNA) that suppressed the pleiotropic effects of this mutation that we have observed in Tetrahymena.

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