SILENCING NEAR tRNA GENES REQUIRES NUCLEOLAR LOCALIZATION*
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Transcription by RNA polymerase II is
antagonized by the presence of a nearby tRNA
gene in Saccharomyces cerevisiae. To test
hypotheses concerning the mechanism of this
tRNA gene-mediated (tgm) silencing, the effects
of specific gene deletions were determined. The
results show that the mechanism of silencing
near tRNA genes is fundamentally different
from other forms of transcriptional silencing in
yeast. Rather, tgm silencing is dependent on
the ability to cluster the dispersed tRNA genes
in or near the nucleolus, constituting a form of
three-dimensional gene control.

Chromatin-mediated transcriptional
silencing has been extensively studied in
Saccharomyces cerevisiae: at the two silent mating
type loci, near telomeres, and in the single cluster
of tandemly repeated ribosomal RNA (rRNA)
genes [1]. Mutations affecting these silencing
forms affect chromatin structure by altering
histone modifications and remodeling. Unlike
other eukaryotes, S. cerevisiae appears to lack
RNA-mediated forms of silencing [2].

Actively transcribed transfer RNA (tRNA)
genescan suppress transcription of nearby genes
by RNA polymerase II (pol II) in yeast [3, 4].
This phenomenon, termed either tRNA gene
position effect [5] or tRNA gene-mediated (tgm)
silencing [6], is independent of the tRNA gene
orientation and does not involve simple steric
blockage of RNA pol II upstream activator sites
[6]. It is dependent on transcription of the tRNA
gene, since mutations in the pol III promoters and
conditional mutations in RNA polymerase III (pol
III) alleviate tgm silencing. The degree to which
this effect suppresses nearby pol II transcription
varies depending the pol II promoter [6].

Unlike other silencing elements, tRNA
genescan occur in large numbers and could potentially influence
neighboring genes, although pol II promoters are
underrepresented near tRNA genes [5]. Notable
exceptions to this are the Ty retrotransposons [5,
7, 8], which appear to have adapted to the
environment and preferentially insert near tRNA
genes. The mechanism of tgm silencing is
unknown, but genetic and cytological data suggest
that it might be linked to spatial organization of
the tRNA genes in the nucleus. The early pre-
tRNA processing pathway and most tRNA genes
associate with the nucleolus in yeast [9, 10], and
tgm silencing is released by a mutation affecting
nucleolar rRNA processing [6].

To explore the mechanism of tgm
silencing we have examined its relationship to
other silencing forms and its dependence on
nucleolar localization.

Materials and Methods
Yeast Strains and Genetic Manipulations - The
strains used for screening gene deletions is
BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0
GAL4 GAL80) and its derivatives, ResGen
Invitrogen Corporation (Carlsbad, California). Deletions affecting tgm silencing were confirmed
by PCR. Growth on selective media was
performed by standard methods except that the
G418 concentration in kan selections was doubled
[11].

Identification of Silencing Suppressors - The
deleted gene strains with plasmid pSUP40[4] were
plated on four different synthetic (S) media for 4-
28 days with either dextrose (D) or galactose and
raffinose (GR) as carbon sources: SD-ura, SD-ura-
his, SGR-ura, SGR-ura-his. No strains grew on
SD-ura-his, as expected (not shown).

Fluorescence in situ Hybridization - The position
of the RNAs and the tRNALeu (CAA) gene family
in the gene deletion strains was detected by in situ
hybridization of fluorescent oligonucleotides as described [9, 10].

RESULTS AND DISCUSSION

We tested specific gene deletions that might have influenced the process for a variety of reasons. Each deletion strain contained a plasmid [4] in which an active SUP4 tRNA gene normally suppresses transcription of a neighboring HIS3 coding region from an artificial GAL1 promoter (Figure 1). Cells are unable to grow in the absence of histidine unless the gene deletion mutations alleviates tgm silencing [6].

The tested gene deletions affect several processes that might affect silencing behavior near tRNA genes. The deletions that do not affect tgm silencing are listed in Table 1 (detailed rationale provided in online supplemental data). None of the gene deletions that affect other forms of transcriptional silencing, histone modification, or chromatin remodeling have any effect on tgm silencing. Because the tRNA genes are nucleolar, it is particularly interesting that several gene deletions that affect Sir2p-dependent and Sir2p-independent rRNA gene cluster silencing (e.g. RP334, RFI, SET1, SIR2, SNF2) [12,13] do not affect tgm silencing. Although these results do not rule out involvement of some aspect of chromatin structure in tgm silencing, they suggest that its mechanism is fundamentally different from most other silencing forms.

A number of gene deletions affecting nuclear morphology, cytoskeleton, and nucleoskeleton were tested to ask whether general defects in nuclear or cellular architecture might affect tgm silencing. This question was of particular interest because tRNA gene localization appears to be important for nearby silencing to occur (see below). None of these tested gene deletions relieved tgm silencing. The remaining category in Table 1 contains non-essential genes that affect rRNA biosynthesis and transport in the nucleus. These were tested because the one gene we previously identified as affecting tgm silencing was CBF5, which encodes a nucleolar rRNA processing enzyme, tRNA pseudouridine synthase [6, 14]. None of the other deletions affecting rRNA processing and transport had any effect on tgm silencing (Table 1). It is possible that the cbf5 allele identified in our previous selections has a specific disruptive effect not mimicked by other deletions of rRNA processing components.

In contrast, four gene deletions that interfered with rRNA gene transcription alleviated tgm silencing (Figure 1 and Table 2). Two non-essential subunits of pol I, Rpa12p [15] and Rpa49p [16], and two subunits of the rRNA gene transcription factors, Rrn10p [17] and Uaf30p [18] gave positive results. Although deletions of these genes cause slow growth, the cells retain recognizable nucleoli (Figure 2), unlike what would be expected from loss of essential pol I subunits [19]. Surprisingly, deletions of two other non-essential pol I subunits, rpa14 and rpa34, do not alleviate tgm silencing even though the slow growth of the strains suggests a partial defect in pol I transcription. It is not clear why this would be true, but it presumably reflects differences in the subunit functions. However, since deletion of four of the six non-essential components of the pol I transcription machinery release tgm silencing, we conclude that some aspect of the early rRNA biosynthetic pathway directly or indirectly influences silencing near tRNA genes.

It is possible that tgm silencing might depend on nucleolar integrity, since a cbf5-1 mutation that releases tgm silencing also slightly disorders the nucleolus [6] and the tRNA genes are largely nucleolar [10]. We therefore tested whether the mutations that alleviated tgm silencing also lost the nucleolar localization of tRNA genes (Figure 2). The positions of the 10 dispersed tRNA<sub>Leu</sub>(CAA) genes family were determined in several strains where genes affecting ribosomal RNA biosynthesis were deleted. Fluorescent oligonucleotide probes were also used to assess the position of the intron-containing, nuclear pre-tRNAs, which have previously been found to be primarily nucleolar [9]. These methods will detect the pre-tRNA transcripts even when distributed throughout the nucleus, but visualization of the tRNA genes depends on clustering of the ten genes [10], since the signal per gene is very low.

In the wild type and Δsir2, Δrpa14 and Δrpa34 control strains, the tRNA<sub>Leu</sub>(CAA) gene family and its pre-tRNA transcripts primarily colocalize with the nucleolus. However, in strains with compromised tgm silencing the nucleolar localization was lost. This included strains with deleted pol I subunits (Δrpa12 and Δrpa49), and missing rRNA gene transcription factors (Δrnn10...
and ΔuaGF30). In these strains the pre-tRNA transcript signal becomes more dispersed in the nucleoplasm and the tRNA gene signal becomes indistinguishable from background fluorescence, the expected result if the genes become dispersed in the nucleus [10]. It therefore appears that the effect of these mutations on tgm silencing might be through compromising the spatial organization of tRNA gene loci.

Spatial positioning of tRNA genes in yeast might be primarily driven by a need to organize the beginning of the tRNA biogenesis pathway, but this does not preclude the possibility that eukaryotic nuclei have developed ways of using the transcriptional side effects of this arrangement. The results presented here support the hypothesis that negative transcriptional regulation near tRNA genes requires subnuclear DNA localization, although it might also require additional mechanisms. Subnuclear positioning of silenced regions appears to be the rule, rather than exception. Telomeres and silent mating type loci in yeast are associated with the nuclear periphery [20, 21], and the ribosomal RNA gene clusters form their own dense, subnuclear structures (nucleoli).

Nucleolar localization might have the side effect of antagonizing pol II transcription for multiple reasons, including scarcity of pol II and appropriate transcription factors or exposure to an unknown antagonist at these locations. The variability in the degree to which tgm silencing is effective on different pol II promoters might then reflect the acquired ability of some pol II promoters to gather factors and pol II or avoid the antagonist(s). For example the Ty retrotransposon promoters, most of which are juxtaposed to tRNA genes, might have activation mechanisms specifically adapted to this environment. In this regard it is interesting that one Ty retrotransposon class, Ty5, inserts preferentially at telomeres and silent mating type loci instead of tRNA genes [24]. Unlike other pol II transcription units, the Ty5 can be expressed in the silencing environment of telomeres, suggesting that Ty5’s insertion preference for these silenced loci is accompanied by a mechanism to overcome those forms of silencing.

It is not clear to what extent these transcriptional effects near tRNA genes will be applicable near pol III transcription units in vertebrates. There is scarce information on which tRNA genes are active in vertebrate development [25], and vertebrates often have large numbers (∼10⁶) of duplicated pol III transcription units (Small Interspersed DNA Elements, or SINEs).[26]. It would be interesting to be able to correlate the activity of these pol III elements with the activity of nearby pol II transcription, and their spatial organization.

**FOOTNOTES**

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**FIGURE LEGENDS**

**Fig. 1** – Deletion of genes affecting rRNA gene transcription relieves tgm silencing. *A*, A reporter construct was maintained in various deletion strains on a low-copy CEN plasmid [4]. The presence of the tRNA gene (*SUP4*) silences transcription of *HIS3* from the GAL1 promoter/UASG unless tgm silencing is weakened. *B*, Yeast strains carrying the indicated gene deletions were tested for silencing by plating serial dilutions on media containing or lacking histidine (SGR-ura or SGR-ura-his). Growth on SGR-ura-his indicates silencing is compromised. Four strains having defective pol I transcription (Δrpa12, Δrpa49, Δrrn10, Δuaf30) showed weakened silencing. Two of the tested genes affecting pol I transcription do not affect silencing in this assay (Δrpa34 and Δrpa14) A sir2 deletion is shown as a representative control for gene deletions that do not affect tgm silencing.

**Fig. 2** – Loss of nucleolar tRNA gene localization in strains that lose tgm silencing. Strains tested in Fig. 1 were probed for the position of the ten tRNA<sup>Len</sup> (CAA) genes and their pre-tRNA<sup>Len</sup> transcripts. Fixed cells were probed with fluorescent oligonucleotides complementary to the pre-tRNA intron or to the non-RNA strand of the tRNA genes. When the ten dispersed tRNA<sup>Len</sup> genes lose the ability to localize to the nucleolus, they become distributed in the nucleoplasm and no fluorescent signal is detected above background [10]. tRNA genes and pre-tRNAs are shown in red, with the nucleolar U14 snoRNA marker in green and the nucleoplasm in blue (DAPI staining).
Table 1 - Gene and ORF Deletions that Do Not Affect tgm Silencing

| Category                                      | Gene names<sup>a</sup>                                                                 |
|-----------------------------------------------|----------------------------------------------------------------------------------------|
| Genes that are known to affect other forms of transcription silencing | ard1; arp5; asf1; bdf1; bre1; gre2; hat1; hst1; hst2; hst3+4; lsm1; mlp1; mlp2; pch2; ptk2; rif1; rif2; rpa34; rpd3; san1; sap30; set1; sif2; sir1; sir2; sir3; sir4; ssh1; tdp1; thp1; tup1; yhc3; yku80 |
| Chromatin establishment and maintenance, remodeling | arp5; arp8; bdf1; bdf2; cdc73; chd1; elp3; est1; est2; fun30; hat1; hda1; hos1; hos2; hst1; htz1; isw1; isw2; itc1; mcm21; nap1; nhp6a; rad5; rad16; rad26; rad54; rdh54; rsc1; rsc2; sap30; set1; snf2; snf3; snf6; spt7; ylr247c |
| Genes that affect nuclear morphology           | arp5; avt4; bre1; dbp3; gre2; hos2; lsm1; pcs60; pho23; ptk2; rot2; rpa34; ssh1; tdp1; thp1; vps13; vps55; yhc3; ybl036c; ybr028c; ydr071c; yjl135w; yjr056c; yol124e |
| Cytoskeleton/nucleo-skeleton proteins         | arp1; arp5; arp6; arp7; arp8; arp9; cin1; cin8; dyn1; gim5; jsn1; kar3; kip3; tub3; mlp1; mlp2; myo3; pac1; sro9; tof1; vik1 |
| Ribosome organization, biogenesis, and transport | cgr1; dbp3; job1; fpr3; fpr4; hmo1; kap123; kem1; nop12; nop13; nop16; nsr1; nup120; rex3; rpa14; rpa34; rrp6; rrp8; sbp1; sgs1; srp40; ssf1; ssf2; ssn1; top1; top3 |

<sup>a</sup> genes may be listed as associated with more than one category; detailed rationale and references provided as online Supplemental Data.
Table 2 – Mutations that Affect tgm Silencing and Ribosomal RNA Biosynthesis

| Gene name | Functions |
|-----------|-----------|
| △rpa12   | Subunit of RNA polymerase I, nucleolar localization |
| △rpa49   | Subunit of RNA polymerase I, nucleolar localization |
| △rrn10   | Component of the Upstream Activation Factor (UAF) complex, activation of RNA pol I; null mutant shows defective RNA pol I transcription and defective RNA pol II silencing at the rRNA gene locus |
| △uaf30   | Component of the Upstream Activation Factor (UAF) complex, nucleolar localization, activation of RNA pol I; null mutant shows defect of RNA pol II transcription silencing at the rRNA gene locus |
| cbf5-1   | rRNA pseudouridine synthase, nucleolar protein found in H/ACA snoRNP complexes; it was previously shown that cbf5-1 disorganizes pre-tRNA location and mildly disorders nucleolus [6] |
Figure 1 - Wang et al.
