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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 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 tRNA genes can suppress transcription of nearby genes by RNA polymerase II (pol II)[1] 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 genes are scattered throughout the genome 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 *his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0 GAL4 GAL80) and its derivatives, ResGen Invitrogen Corp. (Carlsbad, CA). Deletions affecting tgm silencing were confirmed by PCR. Growth on selective media was performed by standard methods except that the G418 concentration in kanamycin selections was doubled (11).

Identification of Silencing Suppressors—The deleted gene strains with plasmid pSUP4o̲ (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 (data not shown).

Fluorescence in Situ Hybridization—The position of the RNAs and the $tRNA^{Leu}$ (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 (Fig. 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 I (see the on-line supplemental data for detailed rationale). 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. RPA34*, *RIF1*, *SET1*, *SIR2*, *SNF2*) (12, 13, 22, 23) 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 I contains non-essential genes that affect rRNA biosynthesis and

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Ⓢ The on-line version of this article (available at http://www.jbc.org) contains a supplemental Table.

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[1] The abbreviations used are: pol II, polymerase II; tgm, tRNA gene-mediated.

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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, rRNA pseudouridine synthase (6, 14). None of the other deletions affecting rRNA processing and transport had any effect on tgm silencing (Table I). 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.

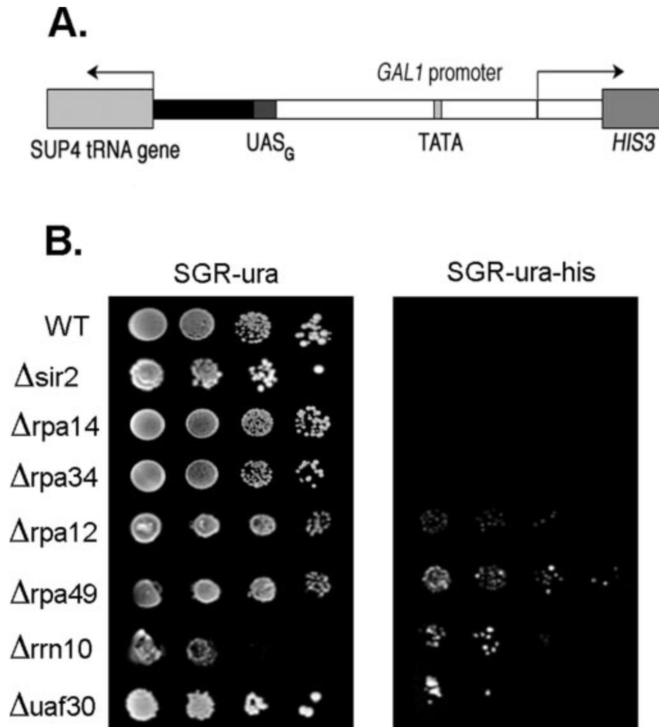

FIG. 1. **Deletion of genes affecting rRNA gene transcription relieves tgm silencing.** *A*, a reporter construct was maintained in various deletion strains on a low copy CEN plasmid (4). The presence of the tRNA gene (*SUP4*) silences transcription of *HIS3* from the GAL1 promoter/UAS$_G$ unless tgm 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 (Δ*rpa12*, Δ*rpa49*, Δ*rrn10*, Δ*uaf30*) showed weakened silencing. Two of the tested genes affecting pol I transcription do not affect silencing in this assay (Δ*rpa34* and Δ*rpa14*) A *sir2* deletion is shown as a representative control for gene deletions that do not affect tgm silencing.

In contrast, four gene deletions that interfered with rRNA gene transcription alleviated tgm silencing (Fig. 1 and Table II). 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 (Fig. 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 (Fig. 2). The positions of the 10 dispersed tRNA^{Leu}(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 Δ*sir2,* Δ*rpa14* and Δ*rpa34* control strains, the tRNA^{Leu}(CAA) gene family and its pre-tRNA transcripts primarily co-localize with the nucleolus. However, in strains with compromised tgm silencing the nucleolar localization was lost. This included strains with deleted pol I subunits (Δ*rpa12* and Δ*rpa49*) and missing rRNA gene transcription factors (Δ*rrn10* and Δ*uaf30*). 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

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TABLE I
Gene and open reading frame deletions that do not affect tgm silencing

Category	Gene names[a]
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*, *ebs1*, *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*, *snf5*, *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, yol124c
Cytoskeleton/nucleoskeleton proteins	*arp1*, *arp5*, *arp6*, *arp7*, *arp8*, *arp9*, *cik1*, *cin8*, *dyn1*, *gim5*, *jsn1*, *kar3*, *kip3*, *tub3*, *mlp1*, *mlp2*, *myo3*, *pac1*, *sro9*, *tof1*, *vik1*
Ribosome organization, biogenesis, and transport	*cgr1*, *dbp3*, *fob1*, *fpr3*, *fpr4*, *hmo1*, *kap123*, *kem1*, *nop12*, *nop13*, *nop16*, *nsr1*, *nup120*, *rex3*, *rpa14*, *rpa34*, *rrp6*, *rrp8*, *sbp1*, *sgs1*, *srp40*, *ssf1*, *ssf2*, *sxm1*, *top1*, *top3*

[a] Genes may be listed as associated with more than one category; for detailed rationale and references provided, see the on-line supplemental data.

TABLE II
Mutations that affect tgm silencing and ribosomal RNA biosynthesis

Gene name	Functions
Δrpa12	Subunit of RNA polymerase I, nucleolar localization
Δrpa49	Subunit of RNA polymerase I, nucleolar localization
Δrrn10	Component of the upstream activation factor 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
Δuaf30	Component of the upstream activation factor 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 (Ref. 6)

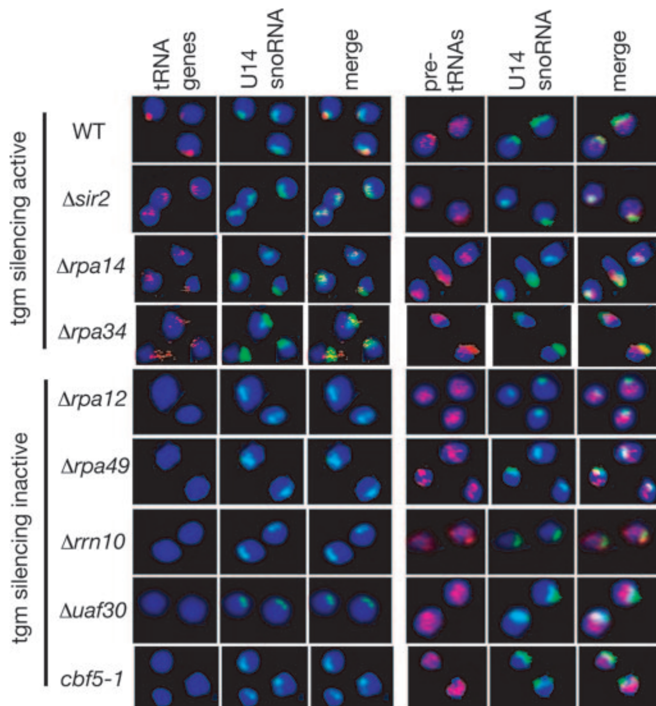

FIG. 2. **Loss of nucleolar tRNA gene localization in strains that lose tgm silencing.** Strains tested in Fig. 1 were probed for the position of the ten $tRNA^{Leu}$ (CAA) genes and their $pre\text{-}tRNA^{Leu}$ transcripts. Fixed cells were probed with fluorescent oligonucleotides complementary to the pre-tRNA intron or to the non-RNA strand of the tRNA genes. When the 10 dispersed $tRNA^{Leu}$ 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* (4′,6-diamidino-2-phenylindole staining).

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 the insertion preference of Ty 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 ($\sim 10^6$) 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.

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