A yeast tRNA^{Arg} gene can act as promoter for a 5' flank deficient, non-transcribable tRNA^{SUP6} gene to produce biologically active suppressor tRNA

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ABSTRACT

In *S. cerevisiae* most tRNA genes are located and expressed as single entities. The tDNA^{Arg}-tDNA^{Asp} pair, however, is transcribed into a dimeric precursor before being processed into two mature tRNA species. The second gene of this pair, tDNA^{Asp}, is totally dependent on the first gene, tDNA^{Arg}, and its promoter components, for homologous *in vitro* transcription. The second gene in the pair is now replaced by the ochre suppressor tDNA^{SUP6-O}, which, by itself, cannot be transcribed because of a nonfunctional 5' flanking region. The tDNA^{Arg}-tDNA^{SUP6-O} was transcribed into a dimeric precursor which was processed to mature tRNA molecules as judged *in vitro* by electrophoretic separation, and *in vivo* by their ability to suppress ochre but not amber yeast mutations. Mutations in the internal promoter of the first gene decreased transcription, both *in vitro* and *in vivo*, of the second - tRNA^{SUP6-O} - gene. Thus tDNA^{Arg} with its 5' flanking region can act as an external promoter for other RNA polymerase III-read genes that are by themselves inactive due to impaired promoter/modulator regions.

INTRODUCTION

Eucaryotic RNA polymerase III (polIII) transcribes genes that code for tRNA, 5SRNA and some small RNAs (1,2,3) from a promoter that resides within the genes themselves as two Internal Control Regions (ICRs) and sometimes also in the 5' flank. The 5'ICR corresponds to part of the dihydrouridine loop and the 3'ICR to the TYCG loop of tRNA. Both intact ICRs seem to be essential in such homologous *in vitro* systems that closely mimic *in vivo* conditions, and even slight changes in ICR sequences may cause marked effects on transcriptional efficiency (4-8).

The requirement for a 5' flanking region seems to be related to the type of cells used as source of enzyme (3). Homologous transcription of silkworm tDNA^{Ala} absolutely required a 5' flank (9,10). We (11) and others (12) have shown that, as in *Drosophila* systems (13), homologous *in vitro* transcription of yeast tRNA genes was dependent, besides the ICRs, on a 5' flanking region the sequence of which varies slightly between position -1 and -10, and can vary somewhat more upstream of position -10. This is in contrast to HeLa or

oocyte systems, where any stretch of DNA can function as a 5' flank for tDNA (14). The transcriptional stop signal, $\geq 4Ts$, is part of the 3' flanking DNA, a region that in some systems appears to also influence the efficiency of transcription (15).

In yeast, tRNA gene pairs are very uncommon. In Schizosaccharomyces pombe the genes for tRNA^{Ser} and tRNA^{Met} are separated by 7 basepairs (16). In Saccharomyces cerevisiae the genes tDNA^{Arg} and tDNA^{Asp} are separated by 10 basepairs, and the gene pair is found in at least two different chromosomal locations (17,18). Their dimeric transcripts are processed by exo- and endonucleases and modifying enzymes to mature tRNA molecules (19,20). Using a homologous S. cerevisiae in vitro system, we have shown (11) that the tDNA^{Asp} in the tDNA^{Arg}-tDNA^{Asp} gene pair was transcribed only as the second member in the pair, although the ICRs of the tDNA^{Asp} have all the requisite consensus sequences. But when preceded by the 5' flank of the tDNA^{Arg}, the tDNA^{Asp} was transcriptionally competent (8). Thus, when occurring as a pair, the tDNA^{Arg} gene and its 5' flanking region can be looked upon as a promoter for the tDNA^{Asp} gene (11).

At least three elements constitutes the yeast pol III promoter function; namely the consensus sequences of the ICRs; the distance between ICRs and the 5' flank. We undertook a study of tRNAs with impaired promoter functions and thus normally not transcribed in vitro or in the cell. This was done by linking nontranscribable tRNA genes to tDNA^{Arg}, using this gene as a means to initiate transcription. Here I present data on the construction of such gene pairs where the second gene originally had a nonfunctional 5' flank. It is shown that dimeric transcripts from these new gene pairs were synthesized in vitro as well as in vivo, that production of processed tRNA^{Arg} and tRNA^{SUP6-o} occurred in vitro and that the processing in vivo to biologically functional tRNA^{Tyr} was manifested as suppression of ochre mutations in yeast cells. Finally it is shown that a mutation in the 3' ICR of the first gene in the pair influenced the level of transcription of both genes in the pair both in vitro and in vivo.

MATERIALS AND METHODS

Bacterial and yeast strains, plasmids and phages. Growth conditions

Bacterial growth in rich media were in YT (Bacto tryptone 8 g/l, Bacto yeast extract 5 g/l, NaCl 5 g/l) at 37°C. Ampicillin resistance clones were selected for at 80 µg/ml ampicillin (rich media) or 50 µg/ml (minimal media). Yeast was grown at 30°C in YPD (yeast extract 10 g/l, peptone 20 g/l

and glucose 20 g/l). Tests of in vivo suppression was done in saltmedium 140, glucose and vitamins (22) supplemented with appropriate aminoacids (tryptophan 20 µg/ml, leucine and lysine 30 µg/ml) and/or bases (adenine or uracil 50 µg/ml).

Yeast strains, plasmids and phages used are listed in Table 1. Phages and vectors were propagated in bacteria (*E. coli* HB101 (23), C800 (24) or JM103 (25)) or in yeast strain *S. cerevisiae*, DH484.

DNA techniques.

Preparations of DNA (> 500 ml cells) followed (23). Small scale preparation (1-5 ml cultures) were as in (26) complemented with a phenol step and two ethanol precipitations. Restriction enzymes were used as suggested by the manufacturers. Degraded DNA was reisolated by phenol and ethanol steps before separating on 5-8 % 1.5 mm polyacrylamidgels (30:1 PA:bisacrylamide) in TBE or in 1/2 TBE (23). pBR322 cut with *Hinf*I was used as marker. DNA-fragments were isolated from the gel by electroelution in 10 or 30 mM Tris-HCl, 1 mM EDTA pH 8.3 at about 200 V for 2-4 h., ethanol precipitated and dissolved in TE-buffer (23). If suitable, carrier RNA (30 µg) was added. DNA ligations using T4 DNA ligase was usually performed at -4°C over night. Bacterial transformation (23) gave frequencies of 10^4 - 10^5 per µg DNA. Yeast transformation followed mainly Hinnens method (27), modified by L. Clarke (28) i.e. treatment with zymolyase or glucylase to get competent yeast cells, gave frequencies of 10^2 - 10^3 per µg DNA. In DNA-DNA and DNA-RNA hybridizations (23) nitrocellulose or HyBond filters were used.

Transcription in vitro

As in the procedure worked out by Engelke (19), intact or restriction fragments of plasmid DNA carrying tRNA genes (0.1-0.2 µg) were incubated in the presence at 9mM MgCl₂, 250 µM ATP, GTP (or UTP), CTP, 25 µM [α -³²P]UTP (or GTP), 1 mM phosphoenol pyruvate, 80-180 mM KCl (optimum varies with the different YNE preparations) and 2 or 4 µl of Yeast Nuclear Extract (YNE) in a total volume of 10 or 20 µl. Incubation was at 30°C for ≤15 min. Reaction was stopped either by proteinase K addition and/or by phenol- ethanol treatment. Transcription products were separated on thin (0.3 mm) 10 or 12 % PA-8 M urea gels and visualized by exposure to X-ray films. Transcription was quantified by Cerenkov counting of excised bands from the gel.

Suppression of ochre mutations in vivo

Yeast cells with several ochre mutations were transformed with plasmid DNA carrying LEU⁺ as selectable marker and tRNA gene pairs, the second gene of which codes for a suppressor tRNA. The level of suppression was quantified

(29) by growing LEU⁺ transformants at 30°C in a complete medium lacking leucine to about 10⁷ cells/ml. The cells were washed and resuspended in water to 10⁹ cells/ml. Then 10 µl of serial dilutions of this suspension was spotted on plates lacking one of the required nutrients. After 3 days and 6 days at 30°C the plates were scored for growth on the spots. The most diluted sample giving confluent growth on the spot was recorded as growth. Furthermore, the degree of red pigmentation on these growth spots was scored Plasmid constructions.

a. Insertion of tDNA^{SUP6-0} instead of tDNA^{Asp} in the tRNA^{Arg}-tRNA^{Asp} gene pair. The ClaI-HpaI fragment from plasmid pJB19f, carrying the tRNA^{Arg}-tRNA^{Asp} gene pair, was further cut with Sau3A giving a fragment containing the upstream 5' flanking region of the gene pair, the tRNA^{Arg} gene, the 10 bp spacer and about 48 bp of the tRNA^{Asp} gene ending in a Sau3A site. This Sau3A site was ligated to a BamHI fragment containing the gene for suppressor tRNA^{Tyr}, preceded by 3 bp of its own 5' flank and followed by about 90 bp of the 3' flank and surrounded by BamHI linkers. This fragment was isolated from plasmid pYSUP6Δ50. Simultaneously, the large ClaI-BamHI fragment of pBR322 was added to this mixture and all these three pieces were ligated together at the same time. One plasmid, pKS14, shown to contain both yeast fragments in correct orientation, was selected for further experiments. Its DNA sequence is given in Fig. 1. Its yeast DNA consists of 5' flank-tDNA^{Arg}-10 bp spacer-53 bp of 5' half of the tDNA^{Asp}+ linker-tDNA^{SUP6(Tyr)} - 3' flank.

The residual part of the tDNA^{Asp} gene was deleted from the fusion by site-directed mutagenesis (4). An EcoRI-BamHI fragment from pKS14 was cloned into M13mp8. One strand of the resulting plasmid M13mp8-at8 was hybridized to the ³²P end-labelled synthetic oligonucleotide 3'GAAACAAAGAGAGGCCATC5', which was complementary to the ten spacer nucleotides and to the ten first nucleotides of the tDNA^{Tyr} in the noncoding strand. After primer extension and ligation, circular labelled DNA was used to transfect competent *E. coli* JM103 cells. One of the obtained deletions, M13mp8Δ33, checked by transcription tests and sequence determination, was selected for further experiments. An EcoRI-BamHI fragment from this clone was cloned into pBR322 thereby creating pKS102. The yeast DNA contained in this plasmid is 5' flank - tDNA^{Arg} - 10 bp spacer - tDNA^{SUP6(Tyr)} - 3' flank (cf Fig. 1).

b. Transfer of cloned tRNA genes into a shuttle vector. The shuttle vector pYCEN3ARS2 (cf Table 1) carries the selectable yeast marker LEU2⁺. It also carries the tRNA^{sup53(Leu)} gene (30), identified by its transcription

Fig. 1. Nucleotide sequence of part of the yeast DNA insert in pKS14.
A compilation of known sequence data (18,21) and of new determination by the Sanger dideoxy method (23) over the new junctions.

products (cf Fig. 2, lane 8), its sensitivity to HaeIII (lane 9) and its insensitivity to MluI (lanes 11 and 12). A PstI-BamHI-fragment from pKS14 (Fig 1) was ligated to the vector pretreated with PstI, BamHI and alkaline phosphatase. The fragment repairing the *bla* gene (Ap^r), which contains the PstI site, also carried the tDNA^{Arg}-part of tDNA^{Asp}-tDNA^{sup6}. One such plasmid, pYCEN3ARS2-511, was selected for in vitro and in vivo experiments. Plasmid pYCEN3ARS2-506 is identical to pYCEN3ARS-511 but isolated by slightly different steps (M. Strobel, pers. com.). In this case a ClaI-BamHI fragment from M13mp8-at8 was ligated to the small ClaI-SalI fragment from pBR322. This ligated DNA was then cut by HindIII to give a HindIII-BamHI fragment, which was then integrated into the pYCEN3ARS2 vector at its HindIII-BamHI sites. This results in a DNA sequence identical to pYCEN3ARS2-511.

The strategy to restore the ampicillin resistance after PstI cuts in the *bla* gene was again used when a PstI-BamHI fragment from plasmid pKS102 was isolated and cloned into pYCEN3ARS2 giving the plasmid pYCEN3ARS2-102. A slightly different mode of cloning - the same as for pYCEN3ARS-506 - gave pYCEN3ARS2-107 (cf Table 1) which is identical to pYCEN3ARS2-102. In the same way a tRNA^{Arg} gene containing a point mutation, C58G in 3' ICR (4) linked to tDNA^{sup6} was inserted in the shuttle vector giving pYCEN3ARS2-108.

TABLE 1
Yeast strains, plasmids and phages used or referred to in this work

Yeast strains	Genotype/characteristics		Reference
<i>S. cerevisiae</i> DH484	<u>a</u> , <u>ψ</u> , <u>ade2-1-o</u> , <u>leu2-3</u> , <u>leu2-112</u> , <u>lys1-1-o</u> , <u>trp5-48-o</u> , <u>ura4-11-a</u> , <u>can^r</u> .		From J. Hicks CSH NY 11724 US, also (21)
<i>S. cerevisiae</i> EJ101	<u>pep4</u>		From E. Jones, see (19)
Plasmid	Vector	Characteristics	Reference
pBR322		Ap ^r Tet ^r	(23)
pJB19f	pBR313	Yeast tDNA Arg-tDNA Asp	(17, 18)
pYSUP6A1u	PBR322	Yeast tDNA SUP6-o(Tyr)	(21)
pYSUP6A1uΔ51	"	Yeast tDNA SUP6-o(Tyr)	(21), Johnson, P, pers. comm.
pYSUP6A1uΔ50	"	Yeast tDNA SUP6-o(Tyr)	(21), Johnson, P. pers. comm.
pKS14	"	Yeast tDNA Arg-5' part of tDNA ^{ASP} -tDNA ^{SUP6-o}	This paper
pKS102	"	Yeast tDNA Arg-tDNA ^{SUP6-o}	This paper
M13mp8			(25)
M13mp8-at8	M13	Yeast tDNA ^{Arg-5'} part of tDNA ^{ASP} -tDNA ^{SUP6-o}	This paper
M13mp8Δ33	"	Yeast tDNA ^{Arg-tDNA^{SUP6-o}}	This paper
pYCEN3ARS2		Shuttle vector <i>E. coli</i> -S. cer., LEU2, tDNA ^{Leu}	From J. Carbon, (35)
pYCEN3ARS2-511	pYCEN3ARS2	Yeast tDNA ^{Arg-5'} part of tDNA ^{ASP} -tDNA ^{SUP6-o}	This paper
pYCEN3ARS2-506	"	Identical to -511, cloned differently	M. Strobel, pers. comm.
pYCEN3ARS2-102	"	Yeast tDNA ^{Arg-tDNA^{SUP6-o}}	This paper
pYCEN3ARS2-107	"	Identical to -102, cloned differently	M. Strobel, pers. comm.
pYCEN3ARS2-108	"	Yeast tDNA ^{ArgC56G} -tDNA ^{SUP6-o} , C56G in 3' ICR of Arg	" and (4)

Fig. 2. Homologous in vitro transcription of yeast tRNA gene pairs. Transcription in vitro with Yeast Nuclear Extract (YNE) was as given in Materials and Methods with [KCl] of 150 mM and [α - 32 P]UTP as added label. Radioactive transcription products formed after 15 min at 30°C were separated on 0.3 mm 12% PA-8M urea gel (38 cm x 28 cm) in 1/2 x TBE run at about 1100V, 30 mA. The gel was exposed to Kodak XAR5 film with intensifier screen at -80°C for 15 h. Lanes 4 and 10 show transcription from plasmids carrying yeast tDNA(Arg-1/2Asp-SUP6(Tyr)) and in lanes 6 and 12 where the residual part of the tDNA^{Arg} had been deleted. Transcription after treatment with MluI, for which there is a site at position +21 within tDNA^{Arg}, are shown in lanes 5, 7, 11 and 13. Transcription from plasmids pJB19f (lane 2) and pYSUP6Alu (lane 15) function as size markers together with tRNA^{Leu} transcripts from the vector pYCEN3ARS2 (lanes 8 and 9). pYSUP6Δ50 = tDNA^{SUP6} in pBR322 deleted of its 5' flank except for 3 bp (lane 14). Blanks (lanes 1 and 16) contain a complete assay mix except for added DNA.

Materials

Restriction enzymes were from Biolabs or BRL, that also supplied T4 DNA ligase, DNA polymerase I (Klenow fragment) and polynucleotide kinase.

Fig. 3. Hybridization of tRNA gene transcription and processing products to yeast DNA

Yeast pJB 19f and pYSUP6 50ΔDNA (cf Table 1) were degraded with restriction enzymes ClaI-HpaI (CH) and BamHI-SmaI (BS) respectively. The tDNA^{Arg} is present only in fragment 1 and the tDNA^{SUP6(Tyr)} only in fragment 2. Different labelled transcription and processing products were hybridized to these DNA fragments. Lane A: Isolated primary transcript from pKS14 (pre-tRNA^{Arg-Asp-Tyr}) reincubated with YNE, resulting processed products separated on 12% PA-8M urea gel, processed transcript (≈90 nt) isolated and used as probe. Lane B: Probe: primary transcript (unspliced untrimmed tRNA^{Tyr}, ≈100 nt) from pYSUP6Alu. Lane C: Probe: processed transcript (pre-tRNA^{Asp} ≈90 nt) from pKS14. Lane D: Probe: transcript (pre-tRNA^{Arg} ≈90 nt) from pJB19f.

Unlabelled nucleotides were from Sigma and [α -³²P]GTP and [α -³²P]UTP (spec. act. 1-10 Ci/mmol) were from Amersham as were the HyBond filters. Polyethyleneglycol 4000 used for yeast transformation was from BDH Chemicals. Synthetic 20-mer was from B. Kaplan, City of Hope Medical Centre, Duarte, California.

RESULTS

The "promoter activity" found in yeast tDNA^{Arg} (11) posed the question if tRNA genes other than tDNA^{Asp} could also be transcribed as a second member in a gene pair preceded by tDNA^{Arg} and then processed into mature tRNAs. As part of our studies on uncoupling of transcription effects of promoter elements from biological activity of the tRNA, we exchanged tDNA^{Asp} for tDNA^{SUP6} creating a gene pair where eventual transcription of tDNA^{SUP6} could be identified in vivo as suppression of ochre mutations. The tDNA^{SUP6} used here is, by itself, non-transcribable in in vitro or in vivo yeast systems due to lack of functional 5' flanking DNA.

The new gene pairs were used to ask 1) if also these tRNA sequences were transcribed as expected into one large multimeric pre-tRNA transcript containing all tRNA sequences simultaneously, 2) if the last gene in the construct was dependent on the first one for transcription in vitro (as expected from our earlier studies), but also in vivo, and 3) if such transcripts as tRNA^{Arg}-tRNA^{SUP6} could be processed into functional tRNA molecules in the cell as well as in vitro, keeping in mind that some of the processing activities acting on the Arg-Asp dimer is still not characterized. The first question, and, to some extent the 2nd and the 3rd, could be answered by in vitro transcription experiments, while the last two also required that the genes (cloned in a shuttle vector) were transcribed and matured within a yeast cell containing mutations that were suppressible.

In vitro transcription of tRNA gene pairs

Crude enzyme extracts made from yeast nuclei catalyze transcription of yeast tRNA genes as well as all processing steps (19) except for complete splicing of pre-tRNA^{Tyr}. In Fig. 2 transcription size markers (18) from pJB19f (Arg-Asp) are the 172 nt primary transcript starting at A-10 (cf Fig 1), the 90 nt pre-tRNA^{Arg}, resulting from an endonuclease cut at the 5' end of tRNA^{Asp} and the 72 nt mature tRNA^{Arg} and tRNA^{Asp} molecules.

When equipped with its own 5' flanking DNA as in the plasmid pYSUP6Alu, the tRNA^{SUP6-o} gene is transcribed (Fig 2, lane 15). The primary transcription product (≈100 nt) is slightly bigger than the trimmed unspliced precursor (90 nt) which, in this system, migrates with the pre-tRNA^{Arg}. The minor part that is spliced is seen as a faint 72 nt band.

When the original 5' flank of tRNA^{SUP6-o} was successively deleted and exchanged for plasmid and BamHI linker DNA, keeping only 23 bp at the original 5' flank, transcription was obtained both in vitro and in vivo (data not shown). When the whole 5' flank except for 3 bp was deleted as in pYSUP6Δ50, the fragment - inserted in a vector or by itself - was unable to support transcription in vitro (Fig. 2, lane 14) or in vivo (data not shown). This fragment was the one that was fused to the tDNA^{Arg}-tDNA^{1/2Asp} genes (see Materials and Methods). The primary in vitro transcripts from such fused tRNA genes (pKS14, lane 4, and pYCEN3ARS2-506, lane 10) were of expected sizes of 243 nt, with transcription starting at A-10, readthrough of tDNA^{Arg}, the spacer, the partial tDNA^{Asp}, the linker and the tDNA^{SUP6-o}. The 90 nt band contained a mixture of pre-tRNA^{Arg} (90 nt) and unspliced tRNA^{SUP6(Tyr)} as shown by hybridizing isolated labelled 90 nt precursors to DNA fragments containing either tDNA^{Arg} or tDNA^{SUP6} (Fig 3, lane C).

Furthermore, both precursors emerged from processing of the primary transcript (Fig 3, lane A). The 72 nt band contained essentially mature tRNA^{Arg} and only minor amounts of mature tRNA^{SUP6(Tyr)} (11,19) due to incomplete splicing.

Except for the tRNA^{Leu} transcript (31) emerging from the vector pYCEN3ARS2, the same general transcription pattern was obtained irrespective of whether the fused genes were cloned into pYCEN3ARS2 (pYCEN3ARS2-506, Fig. 2, lane 10), or whether transcription occurred from an intact plasmid or from a restriction fragment derived from well outside the tRNA genes. Restriction within the tRNA^{Arg} gene at position +21 (lanes 5 and 11), abolished transcription also of the tRNA^{SUP6-o} gene. This occurred in spite of the fact that the tRNA^{SUP6-o} gene contained normal ICRs, known to be important for transcription. Thus, a random DNA sequence of 23 bp upstream of a tRNA gene is not enough to constitute a 5' flank, and some, but not one single, consensus sequences in the 5' flank of the gene are necessary for initiation of transcription.

Transcription of plasmids in which the residual part of the tRNA^{Asp} gene had been deleted gave the expected size of primary transcripts (190 nt) and the same general processing pattern as before (Fig 2, pKS102, lane 6 and pYCEN3ARS2-107, lane 12). In this experiment but not in others, pKS102 (Arg-Tyr) gave two clearly visible additional bands (125 and 110 nt), probably due to premature stops at T-stretches in the beginning of the intron sequences in tDNA^{SUP6}. These bands hybridized tDNA^{Arg} probes (data not shown), they diminished when higher UTP (or GTP) concentrations were used in the assay mix and finally they were abolished when the tRNA^{Arg} gene was cut at position +21 (lanes 7 and 13). A similar "premature stop" band (190 nt) was seen also for Arg-1/2Asp-Tyr constructs (lanes 4 and 10).

Since the second gene is dependent on the first gene of the pair, a mutation in one of the internal control regions - ICRs - in the first gene should lead to decreased transcription of the second gene in the pair. A C58 → G mutation in the TΨCG sequence in the 3'ICR is known to cause a 20-fold decrease in template activity of tDNA^{Leu} (4) and of tDNA^{Arg}-tDNA^{Asp} (8). When introduced into the 3'ICR of tDNA^{Arg} in the tDNA^{Arg}-tDNA^{SUP6(Tyr)} gene pair, the mutation led to a residual template activity of 7 to 18%, mostly recovered as precursor molecules (Fig 4). Besides directly comparing the efficiency of transcription in the different cases, the data were also normalised by using the tRNA^{Leu} transcripts as internal standards, taking into consideration that the assay conditions were chosen so that there was

Fig. 4. Effect on transcription of a mutation in the 3' ICR of the first gene in tDNA^{Arg}-tDNA^{SUP6}(Tyr) gene pairs
Transcription conditions were as in Fig. 2. All 8 incubations contained 0.1 µg of DNA. Total transcription was quantified by scanning of X-ray film (on a shorter exposure than shown here) and by Cerenkov counting of tRNA products excised from the gel. Total transcripts from non-mutated genes (corresponding to lanes 2 and 6) varied in different experiments between 9.500 cpm and 11.600 cpm. The cpm in tRNA^{Leu} transcripts varied between 6 000 and 8 600. Besides direct comparison, all values were also normalized with respect to tRNA^{Leu} transcripts.

no shortage of transcription factors. This was shown by getting about equal incorporation into tRNA^{Leu} irrespective of the plasmid was intact, i.e. transcription from three tRNA genes, or MluI-treated, i.e. transcription from only tDNA^{Leu} (Fig 2, lanes 10-11 and 12-13 and Fig 4). Thus, data from in vitro experiments have shown that tRNA gene pairs were transcribed to large precursor molecules containing sequences corresponding to all present tRNA genes and that mature size molecules are derived only from processing of these dimeric precursors and not from reinitiation of transcription at the second gene.

A tRNA gene pair is processed into mature tRNA^{SUP6} in vivo as judged by ochre suppression

Data shown so far do not prove whether the processed products were biologically active tRNA molecules. All gene pair constructions were chosen

TABLE 2
Suppression of ochre mutations by tRNA^{SUP6-o}(Tyr) transcribed in yeast cells from tDNA^{Arg}-tDNA^{SUP6} gene pairs

Plasmid	Special characteristics	Suppression of mutations in <i>S. cerevisiae</i> DH484 measured as growth					
		on rich medium	-leu	on selective media -tryp	-lys	-ade	-ura
PYCEN3ARS2-511	Yeast tDNA(Arg-1/2Asp-SUP6-o(Tyr))	5 pw	4 pw	3 pw	3 pw	2 w	-
pYCEN3ARS2-102	Yeast tDNA(Arg-SUP6-o(Tyr))	5 pw	4 pw	3 pw	3 pw	2 w	-
pYCEN3ARS2-108	Yeast tDNA(ArgC56G-SUP6-o(Tyr))	5 p	4 pw	2 p	2 p	2 pw	-

Quantification of suppression (see Material and Methods) is given as: strong suppression 5 = growth from 10³ cells, 4 from 10⁴ cells, 3 from 10⁵ cells, 2 from 10⁶ cells and very faint suppression, 1 from 10⁷ cells. It is also given as strong suppression = white colonies (w), pinkish white (pw), pink (p), redish pink (rp) and red (r) = no suppression. Cells were incubated 6 days at 30°C.

to contain as the second member a tDNA^{SUP6-o}, the maturation of which should lead to suppression of ochre mutations in yeast mutant strains. The plasmids used contained tRNA genes for Arg-1/2Asp-TyrSUP6-o(pYCEN3ARS2-511), Arg-TyrSUP6-o(pYCEN3ARS2-102) and ArgC56G-TyrSUP6-o(pYCEN3ARS2-108). DNA from these different tDNA^{Arg}-tDNA^{SUP6} fusions were transformed into a yeast strain carrying three different ochre mutations and one amber mutation. As seen in Table 2 all three gene pairs gave suppression in vivo of the ochre but not the amber (ura4-11) mutations. This proves that biologically functional suppressor tRNA was synthesized from the DNA that was transformed into the yeast cell. Even if in vitro experiments clearly showed that there was no reinitiation of transcription at the tDNA^{SUP6-o} gene in a gene pair (cf Fig 2 the MluI-experiment lanes 11 and 13) reinitiation might still occur in vivo. If this was so, the degree of suppression would be insensitive to such ICR mutations in the first gene that cause a decreased template activity in vitro. Alternatively, if the second gene is only transcribed as the second member in a gene pair with an ICR mutation in the first gene, its suppressor activity is supposed to be less than for a wild type gene pair. Although transcription in vitro of the tDNA^{ArgC56G}-tDNA^{SUP6-o} was reduced to only ~10%, the effect in vivo might not be very pronounced, since once synthesized, a biologically active suppressor tRNA could be re-used in the cell. As seen in Table 2 the suppressor efficiency is lowered in two cases - suppression of ochre mutations in trp 5-48 and lys 1-1 - where more cells were needed to reach the same suppression as with the wild-type gene pair tDNA^{Arg}-tDNA^{SUP6-o}. Using the degree of red pigmentation as a measure of suppression strength, four of the tested sets - ade 2-1, trp 5-48, lys 1-1 and rich medium gave a more pink colour compared to the whiter colonies from

cells suppressed with a wild-type gene pair. Thus a down mutation in the transcriptional control region of the first gene in the pair led to a less efficient suppression, the activity of which is harboured in the non-mutated second tRNA gene in the pair. Thus I conclude that the suppressor activity originates from processing of the large dimeric precursors and not from transcription of the suppressor gene alone.

DISCUSSION

I have shown in this paper that a tRNA gene, tDNA^{SUP6-o}(Tyr), which has intact ICRs but is not transcribed due to an unnatural 5' flanking region, can be transcribed when joined with another tRNA gene, the tDNA^{Arg}, (from the tDNA^{Arg}-tDNA^{Asp} gene pair) to form tDNA^{Arg}-tDNA^{SUP6-o} gene pairs. Both in vitro and in vivo the transcription was initiated by the ICRs and 5' flanking region of the first gene, and resulted in a large dimeric precursor RNA molecule which matured, stepwise, into the two different tRNA molecules. The free passenger gene (tDNA^{SUP6-o}) was biologically active in vivo as shown by its ability to suppress ochre but not amber mutations in yeast cells. The degree of suppression was correlated with the efficiency of transcription of the first gene in the pair.

In *S. cerevisiae* many tRNA genes are preceded by (Py)₃CAACAAA(N)₅ and with an A at the transcription initiation site at position -10 (12,19), which thus may be considered as part of a 5' flank consensus sequence. When the yeast tRNA^{Tyr} gene is inserted in an intact plasmid, in order for transcription to occur, it has in our case to be preceded by about 23 bp of its original 5' flanking DNA, whereas when the gene is in the form of a linear fragment at least 40 bp are needed (11); results largely in agreements with others (12). When the tDNA^{Arg} gene preceding the suppressor gene is cut by restriction enzymes at position +21 (Fig. 2), leaving the suppressor gene (containing normal ICRs) preceded by 60 bp as a 5' flank, the tDNA^{SUP6} is not transcribed. Thus a random stretch of yeast DNA of 40 bp or more cannot function as a biologically active 5' flank in the yeast transcription system. It can thus be concluded that certain sequential constraints - but not one specific sequence - constitute the functional 5' flank of *S. cerevisiae* tRNA genes.

GC-rich sequences, such as those found in BamHI linkers, can in some cases be inhibitory to transcription systems (32,33), an effect which could - although less probable - perhaps explain the inability of our 5' flank deletions to be transcribed. When tDNA^{SUP6-o} was freed of unnatural 5'

flanking sequences and positioned behind the tDNA^{Arg} gene it was transcribed, but only as the second member in a tRNA gene pair.

In the *Xenopus laevis* oocyte system and in the HeLa cell extract system no 5' flanking region, but only the two ICRs, are required for transcription. Initiation of transcription of three nematode tDNA^{Pro} genes fused together head to tail without any linker in between (14), occurred, as expected, at the 5' side of the first gene but also at the junctions between the genes. Thus the preceding tRNA gene did not prevent initiation of transcription at the second gene, nor was it required for transcription of the following genes. This is in contrast to the homologous yeast in vitro system used here, where the second gene in the gene pair is totally dependent on the first gene and its 5' flank for transcription.

As visualized on gels (cf Fig. 2) processing in vitro of the dimeric pre-tRNA resulted in several trimmed RNA products. An early maturation event is the endonuclease cleavage of the dimeric pre-tRNA^{Arg}-tRNA^{Asp} between the spacer and the tRNA^{Asp} (11,19). In the gene pairs transcribed here, the 5' end of the tDNA^{SUP6-o(Tyr)} is in one case connected to a residual part of the tDNA^{Asp}, and in another connected to the 3' end of the spacer. The transcription products mature in both cases to functional tRNA molecules. Thus the "RNase P-like" endonuclease (20) seems in this case to recognize and cut at the 5' end of the tDNA^{SUP6-o(Tyr)} irrespective of the upstream sequences and their conformations. The resultant unspliced tRNA^{Tyr} has a mature 5' end, while the 3' end is processed by either an exonuclease or - as in *Drosophila* (34) - a specific 3' endonuclease. The 5' part of the precursor RNA now contains tRNA^{Arg}, but when transcription was from plasmids pKS14, pYCEN3ARS2-511 and -506, it also contains the residual part of the tRNA^{Asp}. The appearance in the gel of the 92 bp long product (corresponding to the 5' flank-tRNA^{Arg}- the spacer) indicated that the residual part of the tRNA^{Asp} was either degraded by an exonuclease or was recognized by the "RNase P-like" endonuclease. If this was so, the nuclease can recognize half a tRNA molecule and the conformation this sequence takes. The properties of the processing enzymes are not yet clarified, and further studies using gene pairs such as those presented here might help in elucidating the exact recognition mechanisms of for example the "RNase P-like" endonuclease.

The tDNA^{Arg}-tDNA^{SUP6-o(Tyr)} constructions we have made allowed us to investigate transcription from a gene pair both in vitro and in vivo. Processing of the dimeric RNA gave both tRNA^{Arg} and tRNA^{Tyr} in vitro (Fig

2,3) (11). In vivo, tRNA^{SUP6(Tyr)} is synthesized, both when preceded by the tRNA^{Arg}-spacer as well as the tRNA^{Arg}-spacer-part of tRNA^{Asp} as shown by suppression. Whether or not functional tRNA^{Arg} is made in vivo from the gene pair, is at present not possible to prove, since this tRNA is identical to tRNA^{Arg} molecules transcribed from chromosomal monocistronic tRNA^{Arg} genes. However, all in vitro and in vivo data, together, strongly indicate that both tRNAs in the dimeric precursors indeed mature to functional tRNA molecules.

Mutations within an ICR impair the transcription of the gene itself (3). For tandem genes, e.g., tDNA^{Arg}-tDNA^{Asp}(8) and tDNA^{Arg}-tDNA^{SUP6}, such a mutation in the first gene of the pair influences the transcription of both genes in vitro and - as shown for the latter gene pair - also in vivo. In a previously investigated *S. pombe* gene pair containing a suppressor gene, this gene was always the first one in the pair (20). Our constructions with the suppressor gene as the second gene, opened the possibility to study how the biological function - i.e., suppression - of the second gene was influenced by mutations in the first gene. As shown e.g. by introducing a mutation in the ICR of the first gene, which resulted in reduced transcription in vitro and reduced suppression in vivo, no additional initiation of transcription occurred in vitro or in vivo at the start of the second gene in the pair. Although the effects in vivo were small, the results are reproducible and the influence on the biological effect of the second gene in vivo by a mutation in the first gene, is proven. These in vivo effects are of the same order of magnitude as those reported (12) for the single tRNA^{SUP53} gene when it is mutated in the 5' flank in such a way that the transcription in vitro is reduced ~90%. Thus also in vivo the genes are transcribed as a pair. The differences found in suppressibility for different ochre loci is, by itself, very interesting, and opens the possibility to use context effects to investigate e.g. regulation of tRNA synthesis in yeast cells.

The in vitro system used here also transcribed short linear DNA molecules, and could thus be used for specific production of RNA precursors or tRNA molecules with a fairly good efficiency. The conclusion that the first gene in these pairs can lead to transcription of otherwise nontranscribable tRNA genes opens the possibility of using such a system for transcribing in vitro as well as in vivo, nontranscribable genes as a member in a gene pair. If transcription is forced to occur in vivo, i.e. in a yeast cell, it provides an opportunity of studying the fate of a transcription product that normally

is never allowed to be produced in that cell. Thus, the opportunity now exists to uncouple the process of transcription from investigations on biological activity, which might be useful in studies on nucleic acid-protein interactions. One can also speculate on a possible transcriptional event from a structural gene (normally transcribed by polymerase II) coupled to tDNA^{Arg}; whether it is at all transcribed by polymerase III and if so, whether the transcript is stable and processable. If such a system operates, and it should be expected to do so if the DNA fused to the first gene lacks polIII stop signals, it might be used for production of many different types of RNA molecules, both in vitro and in vivo. This might be of special interest as long as no yeast in vitro polII transcription system is operative.

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