

# The use of a synthetic tRNA gene as a novel approach to study *in vivo* transcription and chromatin structure in yeast

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## ABSTRACT

To monitor *in vivo* transcription and chromatin structure of yeast tRNA genes, we constructed a synthetic tRNA gene that can be used as a reporter. Constructs in which this synthetic tRNA gene is combined with different flanking regions can be integrated into the genome as single copies. The artificial tRNA gene is tagged by the insertion of an intron-like sequence that cannot be spliced out from the precursor and transcripts can thus be identified and quantitated. By several criteria, the artificial tRNA gene behaves like a resident tRNA gene. By measuring the accessibility towards DNaseI in chromatin, we found that the artificial tRNA gene exhibits the same characteristic pattern as resident tRNA genes. Three DNaseI-sensitive sites across the transcribed part of the gene and the immediate flanking regions reflect the formation of the stable transcription complex; positioned nucleosomes are observed in the upstream flanking region. We are confident that the system we have established will prove useful for studying regulatory aspects of tRNA gene expression as well as aspects of pre-tRNA processing and splicing.

## INTRODUCTION

In the yeast, *Saccharomyces cerevisiae*, like in other eukaryotes, the production of cellular tRNA species occurs by transcription of the corresponding genes by RNA polymerase III [1] and subsequent processing of the precursor molecules [2]. The formation of a transcription complex requires the initial binding of two specific transcription factors (TFIIIB and TFIIIC) to the tRNA gene [1]: TFIIIC is the first factor to bind to the tRNA gene by interacting with the A and B boxes of the bipartite intragenic promoter; subsequent binding of TFIIIB occurs to the 5' region of the tRNA gene including the transcription start site. In numerous cases (in yeast as well as in other eukaryotic systems), flanking sequences, mainly the 5' flanking regions, also have a role in determining transcription rate. These flanking sequences are generally not well conserved, so that their characteristics and functions are not well understood. The

majority of the studies on modulatory effects have been carried out *in vitro* by the use of cell-free extracts, and no general rules could be derived from these experiments [1].

Generally, multiple copies of the genes for a given tRNA are present [2]. In yeast, like in many other eukaryotes, the individual copies are found scattered throughout the genome and constitute singular transcriptional units [e.g.3]. tRNA gene organization and mechanisms involved in tRNA production have been studied in great detail using *Saccharomyces cerevisiae* as a model system [4]. In yeast, some 12 gene copies exist for each of the major tRNA species, and up to three for the minor ones. About 20% of the nuclear tRNA genes in yeast contain intervening sequences of variable lengths [for recent compilation of *S. cerevisiae* tRNA sequences, see ref.5]; processing of these tRNA precursors occurs by splicing in addition to 5' and 3' processing, in a relatively unordered fashion [e.g.6]. A striking phenomenon for the tRNA genes in yeast is that most of them are found closely associated with sequences derived from transposable elements [e.g.7; 8, and references cited therein]. It has been shown that the 5' flanking regions of the tRNA genes are preferred target sites for the insertion of Ty elements; often transposition occurs into these 'hot-spots', and results in complex patterns derived from sequences of these elements [9–11]. As a consequence, yeast tRNA genes are embedded in different and rather variable sequence contexts.

For particular tRNA genes, short upstream sequence motifs around the transcription initiation site are conserved, and these have been suggested to modulate transcription *in vivo* [1,12,13]. The majority of the tRNA genes, however, lack these motifs and do not exhibit great similarities in their flanking regions. This again underlines the problem of how and to which extent the flanking regions exert modulatory effects on tRNA gene expression. To be able to approach this problem in more general terms, we wanted to develop a system that would permit to measure *in vivo* transcription of a tRNA gene, which had been combined with different flanking regions, and to analyze the underlying chromatin structure concomitantly. This aspect becomes important if one assumes that modulatory effects are not exerted by particular sequences *per se* but that these sequences play a role for the assembly of a particular nucleosomal

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substructure and that the topology of a given tRNA locus will interfere with gene expression. As a basis for such analyses, we have constructed and characterized an artificial tRNA gene which can be used as a unique reporter in yeast cells.

## MATERIALS AND METHODS

### Synthesis of oligonucleotides

tDNA<sup>Syn2</sup> was constructed from two synthetic oligonucleotides representing the complementary strands, each 129 residues in length. The oligonucleotides were prepared by the use of a BioSearch model 8600. After cloning into M13, the correct sequence of tDNA<sup>Syn2</sup> was verified by the Sanger method [14]. Similarly, the 31mer oligonucleotide complementary to the so-called 'pseudo-intron' sequence from tDNA<sup>Syn2</sup> was synthesized and used as a probe in the primer extension experiments.

### Clones, cloning procedures, and transformation of yeast cells

General procedures were according to ref. 15. The yeast shuttle-vectors YIp5 [16], YRp7 [17], and YCp5 [18] have been described earlier. tDNA<sup>Arg(AGG)</sup> was obtained from pBR322 clone H13 [19] and subcloned as a 700 bp *HhaI/BglII* fragment into M13mp10. tDNA<sup>TrpOP</sup> [20] and tDNA<sup>Leu3</sup> [13] are described in the references. For cloning tDNA<sup>Syn2</sup>, the separate oligonucleotide strands were annealed and ligated into appropriate vectors. The following constructs were made: (i) M13-tDNA<sup>Syn2</sup> by blunt-end insertion of tDNA<sup>Syn2</sup> into M13mp10/*SmaI*; (ii,iii) YCp5-tDNA<sup>Syn2</sup> and YRp7-tDNA<sup>Syn2</sup> by insertion of the *HindIII* fragment from M13-tDNA<sup>Syn2</sup> carrying tDNA<sup>Syn2</sup> into YCp5/*HindIII* and YRp7/*HindIII*, respectively. (iv) YIp5-tDNA<sup>Syn2</sup> by recloning the *Sall/HindIII* fragment from M13-tDNA<sup>Syn2</sup> carrying tDNA<sup>Syn2</sup> into YIp5/*HindIIIxSall*.

YIp5-tDNA<sup>Syn2</sup> was used to insert various yeast DNA fragments upstream of tDNA<sup>Syn2</sup> into the *HindIII* site; the following constructs resulted: (v) 80-tDNA<sup>Syn2</sup>: insert, a 1 kb *HindIII* fragment from pY80 [21], representing the 3' flanking region of a tRNA<sup>Glu3</sup> gene; (vi) 5dr-tDNA<sup>Syn2</sup>: insert, a 590 bp *BglII/PvuII* fragment from pY109 [22]; (vii) 3ds-tDNA<sup>Syn2</sup>: insert, a 870 bp *BglII* fragment from pY109 [22] containing a tRNA<sup>Trp</sup> gene, 230 bp downstream from Ty109 (unpublished); (viii) PHO-tDNA<sup>Syn2</sup>: insert, the 1.1 kbp *ApaI/BstEII* fragment from YCpPHO5 [23] containing the promoter region of the *PHO5* gene; (ix) 922-tDNA<sup>Syn2</sup>: insert, the 1440 bp *HindIII* promoter fragment of the *TYRI* gene from cosmid c922 [18] in inverted orientation, with a delta sequence integrated 459 bp upstream of tDNA<sup>Syn2</sup>.

Yeast transformation was by the lithium acetate procedure of Ito et al. [24]; *S. cerevisiae*, strain DBY746 ( $\alpha$ , his3-1, leu2-3, leu2-112, ura3-52, trp1-289<sub>a</sub>; D.Botstein) was used throughout. Transformants were selected on minimal medium with amino acids but without uracil, and identified by Southern blotting using appropriate DNA probes.

### *In vivo* labelling, isolation and analytical gel electrophoresis of RNA

50 ml cultures of yeast cells were grown overnight in low-phosphate medium [25] supplemented with carrier-free [<sup>32</sup>P]phosphate (5 $\mu$ Ci/ml). RNA was isolated by extraction of the cells with phenol at room temperature and ethanol precipitation, and separated by electrophoresis on polyacrylamide gels (10% in 8 M urea) [25].

### Filter hybridization analysis of RNA

For filter hybridization, unlabeled RNA was prepared from yeast cells as described above, 1–5  $\mu$ g RNA separated on preparative polyacrylamide gels (10%, 8 M urea, 1 mm thick) and transferred onto Nylon membranes (Gene Screen plus, NEN) by the use of Hoefer Transphor unit with TEB buffer (0.1 M Tris, 0.08 M borate, 0.1 mM EDTA). The filters were baked in an oven at 80°C, 5–10 Torr for 60 min. Hybridization was performed in sealed plastic bags with a probe of nick-translated M13-tDNA<sup>Syn2</sup> in 5 $\times$ SSC, 50 mM sodium phosphate, pH 6.5, 1 $\times$ Denhardt solution, 1% SDS, 150  $\mu$ g/ml sheared salmon sperm DNA, 50% formamide, at 42°C overnight. The filters were washed twice with 2 $\times$ SSC, 0.1% SDS at RT, followed by one treatment with 2 $\times$ SSC, 1% SDS at 56°C, dried and radioautographed.

## RESULTS

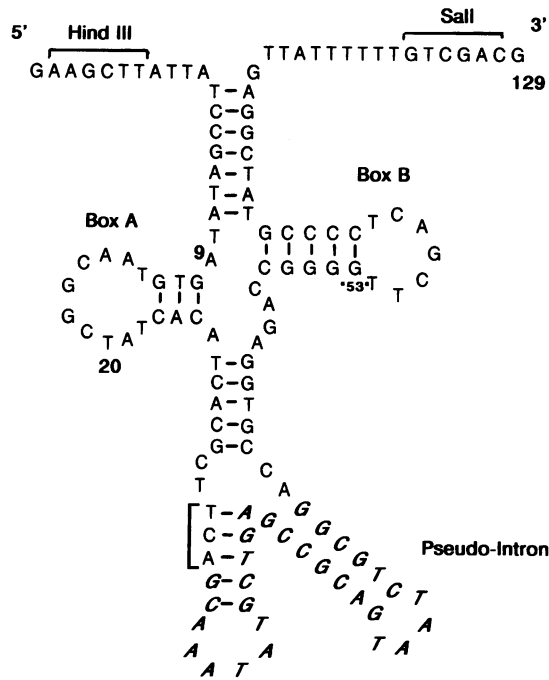
### Construction and cloning of tDNA<sup>Syn2</sup>

An artificial tRNA gene that can be used as a reporter in yeast has to fulfill several criteria. (i) The gene should be transcribed to the same extent as resident tRNA genes. (ii) The transcripts should be stable and amenable to 5' and 3' processing. (iii) The precursors have to be discriminated from the rest of cellular tRNA products in order to allow their quantification, with reference to an internal standard. (iv) Constructs should be available that allow the introduction of a single copy into the cells and, at the same time, to establish variants with different flanking regions.

As a first approach, we synthesized a gene (tDNA<sup>Syn1</sup>) that would render a completely novel tRNA as a product. In agreement with the canonical structural parameters for eukaryotic tRNAs [5], all invariant or semi-variant positions were kept in tDNA<sup>Syn1</sup>, whereas the non-variant positions were modified but necessary base-pairing retained. However, expression of tDNA<sup>Syn1</sup> constructs after integration into yeast cells or micro-injection into *Xenopus* oocytes was too low to make this a useful reporter (data not shown).

We therefore followed a different concept in constructing another artificial tRNA gene, which we named tDNA<sup>Syn2</sup> (Figure 1). The body of this gene is formed by the sequence of the yeast tRNA<sup>Glu3</sup> gene. We have recently characterized most of the members of the corresponding multi-gene family [8]; none of these copies contains an intron sequence. The anticodon triplet in tDNA<sup>Syn2</sup> was changed to T-C-A (for the *opal* codon UGA), and 3' to it a 31 bp sequence was inserted which mimics the presence of an intervening sequence. However, this nucleotide sequence and the position of its 'insertion' was designed in such a way that it should not be spliced from the precursor and thus allow the use of the corresponding deoxyoligonucleotide as a specific probe. For simplicity, we call this inserted sequence a 'pseudo-intron'. Furthermore, to allow for directional cloning, a *HindIII* site was placed in front of the gene and a *Sall* site attached 3' to it after a runs of six T's, which are known to form the transcription terminator for nuclear tRNA genes [1]; the nucleotide sequence of the 5' flank contains purine residues which serve as initiation sites in transcription of yeast tRNA genes [26,27]. We will demonstrate the validity of these features in the following sections.

The two complementary strands of tDNA<sup>Syn2</sup>, each 129 nucleotides in length, were synthesized on an automatic device, annealed and cloned into M13mp10 to yield the plasmid M13-tDNA<sup>Syn2</sup>. The correct insertion and sequence of the insert



**Figure 1:** Nucleotide sequence of tDNA<sup>Syn2</sup> ('non-coding RNA strand') shown in the clover-leaf form. A HindIII and a Sall site, respectively, were attached to allow cloning of the tDNA. 'BoxA' and 'BoxB' refer to the canonical internal recognition signals for RNA polymerase III [2]. The numbers designate specific nucleotides in the standard tRNA nomenclature [2]. The beginning and the end of the inserted sequence (pseudo intron) are indicated by the arrows. The potential anticodon is bracketed.

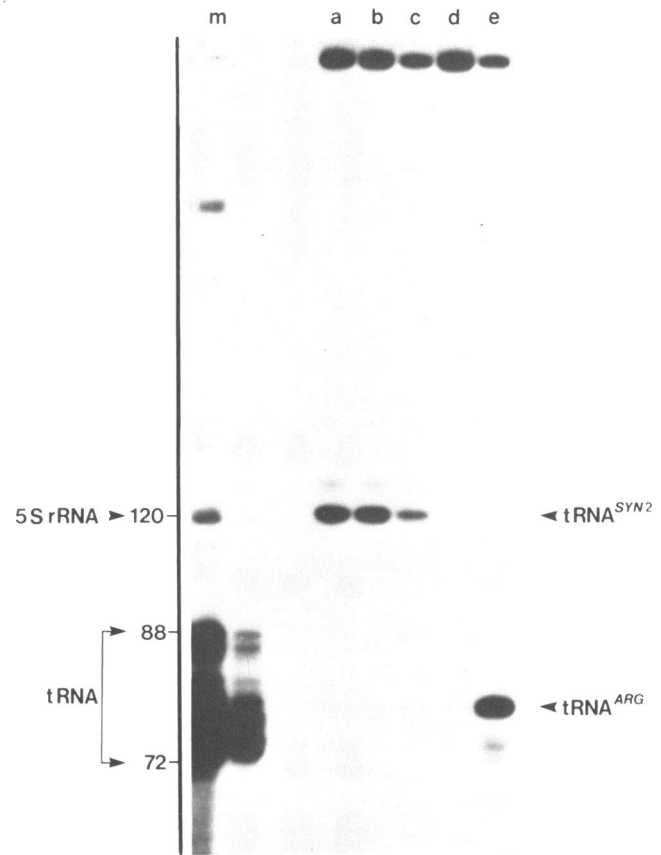
was verified by the Sanger method [14]. The insert from M13-tDNA<sup>Syn2</sup> was then used to construct several yeast shuttle-plasmids carrying tDNA<sup>Syn2</sup> (see Materials and Methods).

#### tDNA<sup>Syn2</sup> is expressed in *Xenopus* oocytes

Micro-injection into *Xenopus* oocytes can be used to test transcriptional activity of a tRNA gene construct [28]. We took advantage of this procedure to demonstrate (Figure 2) that M13-tDNA<sup>Syn2</sup> is transcribed in this heterologous system to the same extent as a native yeast tRNA gene (tDNA<sup>Arg</sup>) on a plasmid: this is obvious by comparing lanes a and e in Figure 2. M13-tDNA<sup>Syn2</sup> yields a product compatible in size with a tRNA<sup>Syn2</sup> precursor which is not spliced.

#### Specific precursors are produced from tDNA<sup>Syn2</sup> in yeast cells

To demonstrate that tDNA<sup>Syn2</sup> is expressed *in vivo* like a resident tRNA gene, we transformed yeast cells with appropriate tDNA<sup>Syn2</sup> constructs. YCp5-tDNA<sup>Syn2</sup> carrying the *ARS1* element and *CEN6* [30] was employed as a 'single copy' self-replicating plasmid, and YRp7-tDNA<sup>Syn2</sup> as a multi-copy self-replicating plasmid for transformation. Transformants were grown in the presence of <sup>32</sup>P-phosphate, RNA was extracted and fractionated by polyacrylamide electrophoresis. The results of Figure 3 show that the transformants (lanes a to c) produce a tRNA<sup>Syn2</sup> precursor which has the same size as the one observed in the oocyte system (lane m) and, by extrapolating from the marker sizes, is 103–104 nucleotides in length. This conforms to the size of a molecule which retains the 'pseudo-intron' (i.e. no splicing occurred) but appears to be correctly processed at its 5' and 3' extensions.

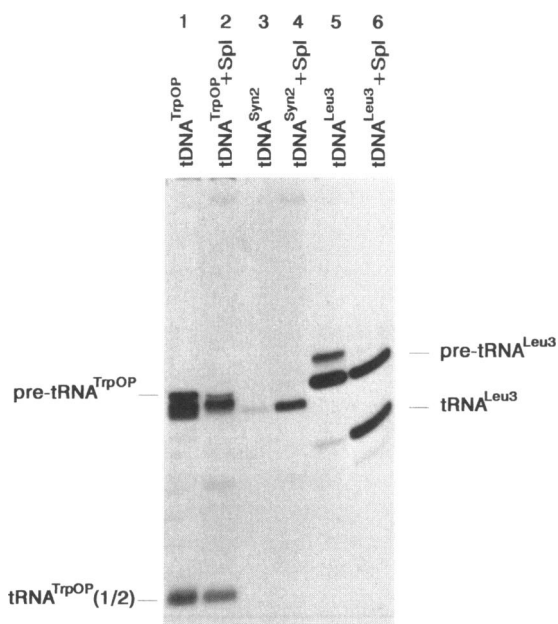


**Figure 2:** Analysis by polyacrylamide gel electrophoresis of products synthesized from tDNA templates in *Xenopus* oocytes. All methods used in this procedure were described previously [29]; extracts from 5 oocytes were applied per lane. The following templates were used: a to c, M13-tDNA<sup>Syn2</sup> (8, 6, and 2 ng/oocyte, respectively); d, none; e, tDNA<sup>Arg</sup> (8 ng/oocyte). m, <sup>32</sup>P-labeled soluble yeast RNA [34]. Electrophoresis (see Materials and Methods) was performed for 36 hrs at 4°C and 10 Volts/cm on 1 mm gels.

#### Primer extension shows correct processing of tDNA<sup>Syn2</sup> *in vivo* transcripts

Bulk RNA containing the tRNA<sup>Syn2</sup> precursor was isolated from cells transformed with YCp5-tDNA<sup>Syn2</sup>, the 31mer dideoxy-oligonucleotide complementary to the 'pseudo-intron' sequence annealed, and this primer extended by the use of reverse transcriptase and labelled dNTPs. The cDNA was then analyzed on a polyacrylamide gel (Figure 4, lanes 1 and 2) using the products from sequencing reactions of M13-tDNA<sup>Syn2</sup> as a reference (Figure 4, lanes G,A,T, and C). Two prominent signals (marked '+1' and '+21' in lanes 1 and 2) indicate that reverse transcription of the tRNA<sup>Syn2</sup> precursor is interrupted at these positions. This result leads to the following conclusions: (i) The tRNA<sup>Syn2</sup> precursor starts with the same nucleotide (U) as expected for a precursor molecule correctly processed at its 5' end. Transcriptional initiation of tDNA<sup>Syn2</sup> *in vitro* occurs 10 to 11 nucleotides upstream from the 5' end of the tRNA<sup>Syn2</sup> precursor (data not shown). Hence, we conclude that the transcripts of tDNA<sup>Syn2</sup> are processed in the same way as the native tRNA gene transcripts. (ii) The tRNA<sup>Syn2</sup> precursor was not spliced and retains the 'pseudo-intron'; otherwise, it could not have served as a template in the primer extension reaction. (iii) tRNA<sup>Glu3</sup> has a dihydrouridine at position 20 [31] derived from a U in the tRNA<sup>Glu3</sup> precursor. From the 'strong stop'



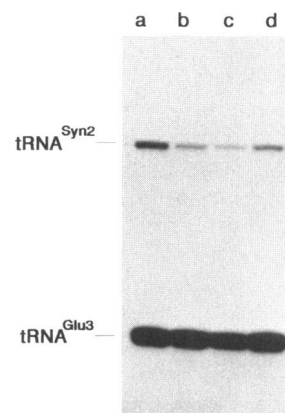


**Figure 5:** Analysis of the products from *in vitro* transcription and splicing reactions using various tDNAs as templates. The combined reactions for transcription of tDNA and splicing of the precursor tRNA were carried out as described in ref. 25. Lanes are marked according to the templates used. In the +Spl lanes (lanes 2, 4 and 6), the products were obtained from reaction mixtures supplemented with transcription factors plus splicing extracts; in the control lanes (lanes 1, 3 and 5), splicing extracts were omitted. tRNA<sup>TrpOP(1/2)</sup> refers to non-ligated half-molecules.

an internal standard. Figure 6 shows an example of this procedure with a set of four different tDNA<sup>Syn2</sup> constructs. It is obvious that the *in vivo* expression of the gene depends on the nature of the upstream flanking sequences. A systematic study of the *in vitro* and *in vivo* effects of defined 5' flanking sequences on tDNA<sup>Syn2</sup> expression will be presented elsewhere.

### The chromatin organization of integrated tDNA<sup>Syn2</sup> is like that of native tRNA genes

It is well established that in several cases specialized chromatin structures are associated with active genes [35, for review]. Experiments using micrococcal nuclease and DNaseI to probe hypersensitive sites have indicated that similar features may apply to tRNA genes in *Drosophila* [36]. However, no direct correlation between tRNA gene expression and a particular chromatin structure has been demonstrated to date. To verify that tDNA<sup>Syn2</sup> is an appropriate system for this type of study, we used the indirect end-labelling procedure to map DNaseI-sensitive sites [37] in some tDNA<sup>Syn2</sup> derivatives and in tDNA<sup>Glu4</sup> (a singular intronless yeast gene) [38] (Figure 7). Panel A of Figure 7 shows the pattern obtained for tDNA<sup>Glu4</sup> chromatin with two DNaseI concentrations (lanes 1 and 2, respectively); naked tDNA<sup>Glu4</sup> served as a control (lane f). Panel B of Figure 7 gives a similar experiment for 3ds-tDNA<sup>Syn2</sup>. This construct carries tDNA<sup>Syn2</sup> and an additional tDNA<sup>Trp</sup> 170 bp upstream of tDNA<sup>Syn2</sup>, in the same orientation. Originally, tDNA<sup>Trp</sup> is located near Ty109 [22, and unpublished results] and contains a 34 bp intervening sequence comparable in length to the 'pseudointron' in tDNA<sup>Syn2</sup>. Panel C of Figure 7 shows the patterns obtained for 922-tDNA<sup>Syn2</sup> chromatin (lane 1) and naked 922-tDNA<sup>Syn2</sup> (lane f). In all cases (as high-lighted by the same



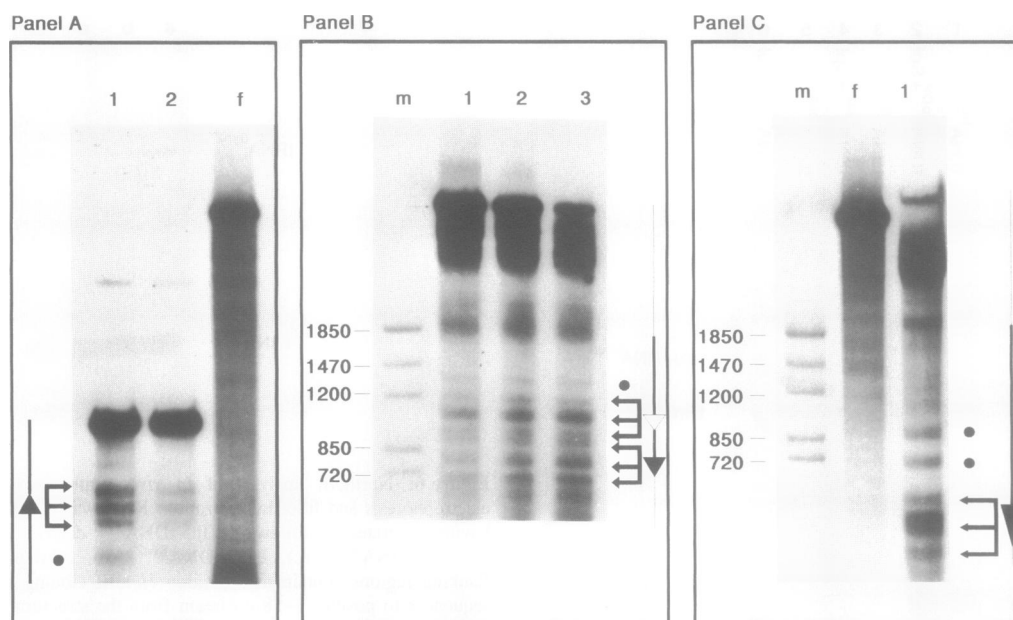
**Figure 6:** Northern analysis of *in vivo* synthesized Syn2-tRNAs by gel electrophoresis and filter hybridization. RNA was isolated from transformants having integrated the following YIp5-tDNA<sup>Syn2</sup> derivatives: (a), 5dr-tDNA<sup>Syn2</sup>; (b), 3ds-tDNA<sup>Syn2</sup>; (c), PHO-tDNA<sup>Syn2</sup>; (d), 80-tDNA<sup>Syn2</sup>. The upstream flanking regions contain the common HindIII cloning site and the following sequences to position -58 upstream from the structural part of tDNA<sup>Syn2</sup>:

ttactaataaggctgtattctactctctcttatatagaataagagatcAGCTTATTAT- (a)  
tgtttggcgactatttcgatggcgtttccaggaaagtataatagatcAGCTTATTAT- (b)  
gtccctgttttgaagagatcgacatgcacaaattatcaaatgtgtcacAGCTTATTAT- (c)  
taatgcaaacactttttaaggatcgactaagggttttcgacacattcaAGCTTATTAT- (d)

symbol), a characteristic band triplet is observed which arises from three sensitive sites within the tRNA gene region; sequences between these sites are protected from nuclease attack. Protected segments include the A and B boxes, and extend 30–40 bp upstream and 10–20 bp downstream from the structural body of the tRNA gene, respectively. The resolution of the bands with an error of 10 bp. Note that the relative positions of the three bands and the spacings between them are different, due to the fact that tDNA<sup>Syn2</sup> and tDNA<sup>Trp</sup> contain intron sequences while tDNA<sup>Glu4</sup> does not, and that different hybridization probes were used. Depending on the construct, further DNaseI-sensitive sites at a more or less regular interval are monitored within the 5' flanking region. The spacings between the corresponding bands (approximately 160 bp) reflect an array of positioned nucleosomes [40]. By contrast, the spacings between the three DNaseI-sensitive sites within the tRNA gene region clearly do not reflect such an array. The most obvious interpretation is that it is the stable complex formed between tDNA and transcription factors (TFIIIC/TFIIIB) [1] which gives rise to the characteristic band triplet. In any case, our results demonstrate that the basic chromatin organization of tDNA<sup>Syn2</sup> is equivalent to that of native yeast tRNA genes.

### DISCUSSION

Our experiments describe a novel approach to use an artificial tRNA gene in place of a resident single-copy tRNA gene to monitor directly *in vivo* transcription and chromatin organization in yeast. As the gene for a completely 'novel' tRNA (tDNA<sup>Syn1</sup>) did not fulfill our requirements, we followed a different strategy in constructing tDNA<sup>Syn2</sup>: the body of this artificial gene corresponds to a tRNA<sup>Glu3</sup> but is tagged by the insertion of an intron-like sequence that cannot be spliced out from the precursor. tDNA<sup>Syn2</sup> proved to meet the expected requirements and in all our experiments behaved like a resident tRNA gene. It can be combined with different flanking regions and can be placed into



**Figure 7:** Chromatin structure of tDNAs. All methods used for the isolation of yeast nuclei, nuclease digestion, gel electrophoresis, and hybridization with appropriate probes (labelled by the random primer method [39]) were according to those described in ref.40. Screen Plus membranes (NEN) were used for Southern transfer. Panel A, tDNA<sup>Glu4</sup>; panel B, 3ds-tDNA<sup>Syn2</sup>; panel C, 922-tDNA<sup>Syn2</sup>. m, marker DNA fragments of defined sizes. f, control digestions (0.02 units DNaseI/ml) of free DNA; plasmid DNA pYH55-Glu4 [38] carrying the tDNA<sup>Glu4</sup> region (panel A), plasmid DNA 922-tDNA<sup>Syn2</sup> (panel C). Different concentrations of DNaseI were used to digest isolated nuclei: 3 units/ml (panel B, lane 3), 1.2 units/ml (panel A, lane 1; panel B, lane 2; panel C, lane 1), 0.6 units/ml (panel A, lane 2). After digestion of the nuclei, DNA was isolated and cut with an appropriate restriction nuclease to create a defined 3' end: *DraI* (panel A); *SryI* (panel B); *NruI* (panel C). The probes used to monitor the DNaseI-sensitive sites in the different constructs were the following: 250 bp *SryI/DraI* fragment from pYH55-Glu4 [38] (panel A); 400 bp *NruI/SryI* fragment from pBR322 (panel B); 330 bp *Sall/NruI* fragment from pBR322 (panel C). The tRNA genes are represented by the triangles (filled triangles, tDNA<sup>Syn2</sup>; open triangle, tDNA<sup>TTP</sup>). Thick lines correspond to yeast DNA sequences, thin lines to YIp5 DNA sequences, respectively. The characteristic band triplet (see text) is marked by three linked arrows. Sensitive sites between positioned nucleosomes are indicated by circles.

different genetic contexts; no resident tRNA gene has to be modified or replaced prior to the measurements. Transcription of tDNA<sup>Syn2</sup> is as efficient as that of an average native tRNA<sup>Glu3</sup> gene. tDNA<sup>Syn2</sup> transcription starts and stops at similar positions as observed for native tRNA genes. tDNA<sup>Syn2</sup> transcripts are correctly processed at the 5' and 3' extensions as inferred from the primer extension experiment, whereas the 'pseudo-intron' is completely retained in the tRNA<sup>Syn2</sup> precursor. This precursor is the only stable molecule obtained after expression of tDNA<sup>Syn2</sup>. It therefore fulfills the requirements we implicated in its design: tRNA<sup>Syn2</sup> precursor can be discriminated from any other product of endogenous tRNA gene expression. The great advantage of the use of tDNA<sup>Syn2</sup> compared to similar approaches employing modified suppressor tRNA genes [13,41] is that its product is directly and easily quantitated with reference to an internal standard.

Furthermore, the analysis by DNaseI of the chromatin structure demonstrates that tDNA<sup>Syn2</sup> exhibits the same characteristic pattern of DNaseI-sensitive regions as resident tRNA genes. We interpret this 'in vivo-footprint' to be due to the stable complex formed between tDNA and components of the polymerase III transcription machinery, which protects defined regions from nuclease attack and leaves adjacent regions accessible to DNaseI digestion. This interpretation is in good agreement with several other findings. Earlier analysis by DNaseI, micrococcal nuclease and S1 nuclease of *Drosophila* tRNA genes gave a first indication of regularly arranged sensitive sites within these regions [36]. Several studies [1 for review, 42] have documented that transcription factor TFIIC is necessary for the assembly of a stable tRNA gene transcription complex. Recent genomic

footprinting experiments [43] have shown that a region up to -40 of yeast tRNA genes is protected in isolated yeast nuclei, suggesting that the stable transcription complex in addition to TFIIC is made up of one or more distinct transcription factors, perhaps including TFIIB. Recent *in vitro* studies [27,12] clearly demonstrated that TFIIB is the central transcription factor of yeast RNA polymerase III, binding to DNA upstream of the transcription start site of tRNA genes (-40 to -8) in a TFIIC-dependent reaction prior to RNA polymerase III. TFIIB remains tightly bound to DNA during transcription and can direct multiple rounds of transcription, whereas TFIIC can be stripped off without loss of transcriptional activity. Following the model proposed by Kassavetis et al. [12] and Huibregtse and Engelke [43], the chromatin pattern observed in our experiments is due to a stable complex with at least TFIIB and TFIIC bound to the tDNA template. As the nuclei for our experiments were isolated from exponentially growing cells, the transcription complex might also contain polymerase III, but our data do not provide any information concerning this possibility.

The fact that tDNA<sup>Syn2</sup> transcripts are correctly processed indicates that these molecules will adopt a proper three-dimensional structure [32]. The result of the primer extension experiment even suggests that certain modifications of the tRNA<sup>Syn2</sup> precursor, like formation of dihydrouridine, can occur. A canonical three-dimensional structure of the tRNA<sup>Syn2</sup> precursor might also be a prerequisite to make it a stable molecule; it has been suggested that precursors adopting 'false' three-dimensional structures might be degraded and thus eliminated from the cell. It should be mentioned here that we have tested the stability of the tRNA<sup>Syn2</sup> precursor

experimentally: RNA was isolated from yeast cells grown to various stages and the rate of tRNA<sup>Syn2</sup> precursor vs. tRNA<sup>Glu3</sup> was determined. The ratio remained constant (data not shown).

In constructing tDNA<sup>Syn2</sup>, we have modified those features which appear to be most critical for splicing of the RNA precursor, namely the sequences around the 5' and 3' proximal splice junctions, although no general rules have been defined up to now [44]. Other features such as the potential of the anticodon/intron sequence to adopt a particular base-paired structure were maintained. In contrast to naturally occurring pre-tRNAs, we introduced one extra C residue 3' to the anticodon which by interaction with a G residue in the intervening sequence prolongs the extended anticodon stem by one base-pair. This modification alters the canonical 5' proximal splice junction. The 3' proximal splice junction normally resides in a loop at least 3 bases in length [32,45], although deviations from this rule have been reported [34,46]. Our construct can adopt a potential base-paired structure which would leave two bases (ApC) unpaired in this loop. Additionally, it is known that sequences within the mature domain affect the recognition of the precursor by endonuclease [e.g.44,47]. However, it is difficult to sort out all possible effects. By all criteria, the tRNA<sup>Syn2</sup> precursor is resistant to splicing endonuclease activity, both *in vitro* and *in vivo*. These features of tRNA<sup>Syn2</sup> precursor might prove useful for studying aspects of pre-tRNA processing and splicing.

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