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FURTHER CHARACTERIZATION OF $tRNA^{TRP}$ GENES OF *SACCHAROMYCES CEREVISIAE*

BY

MOHAMMAD ALI RIAZI

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

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To whoever this may concern,

This letter grants permission to Mr. M. A. Raizi to print a figure as part of his Master's thesis which was originally published as Figure III-1: Primary sequence and possible secondary structures of the *Saccharomyces cerevisiae* $tRNA^{Trp}$ precursor, page 125 of A. L. Atkin's Ph.D. thesis. A. L. Atkin's thesis is entitled *In vivo* and *in vitro* characterization of *Saccaromyces cerevisiae* $tRNA^{Trp}$ suppressor constructs, and was published in Spring 1992 at the University of Alberta.

Yours sincerely,

Audrey L. Atkin

Dr. Audrey L. Atkin

UNIVERSITY OF ALBERTA

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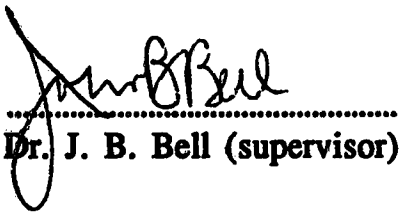
The undersigned certify that they have read, and recommend to the faculty of graduate studies and research for acceptance, a thesis entitled FURTHER CHARACTERIZATION OF $tRNA^{TRP}$ GENES OF *SACCHAROMYCES CEREVISIAE*, submitted by MOHAMMAD ALI RIAZI in partial fulfilment of the requirements for the degree of MASTER OF SCIENCE.



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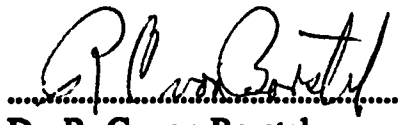

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This thesis is dedicated to Azam for her love, support, and patience through difficult moments.

ABSTRACT

Two goals were pursued in the present work. First, attempts were made to construct an intronless ochre suppressor $tRNA^{Trp}$ gene. The ability of the construct to function as an ochre suppressor was studied *in vivo* by transforming different *Saccharomyces cerevisiae* strains harbouring certain ochre mutations, and then assaying the suppression on selective media. While the intron-containing counterpart of this gene proved to be a nonfunctional ochre suppressor, even when present in high copy numbers in yeast, the *in vitro*-constructed intronless $tRNA^{Trp}$ gene could suppress the tested ochre mutations. This result supported models emphasizing the importance of the intron-anticodon stem loop binding in the splicing of the pre-tRNA transcripts. These interactions are supposedly disrupted after a CCA to TTA change in the anticodon of the putative suppressor $tRNA^{Trp}$ gene. An intronless gene would not have this problem since the need for splicing is abolished. The other interesting aspect of this work was the enhancing effect of an extrachromosomal factor (ψ^+) present in some yeasts. The ochre suppressor ability of the *in vitro*-constructed $tRNA^{Trp}$ gene to suppress one of the tested ochre mutations *(lys1-1)* was significantly increased in the presence of this factor, and it was reduced when this extrachromosomal factor was eliminated. In the second part of this work the 5' and 3' flanking regions of the six $tRNA^{Trp}$ genes present in haploid *Saccharomyces cerevisiae* were studied. These regions were amplified using an inverse PCR technique. Finally, a number of them were sequenced.

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ABBREVIATIONS

AAR	Aminoacyl-tRNA synthetase
bp	Base pair
Ci	Curie
cpm	Counts per minute
DMSO	Dimethyl sulfoxide
dNTP	Deoxynucleoside triphosphate
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
5-FOA	5-Fluoroorotic acid
IPP	Δ^2–Isopentenyl pyrophosphate
IPTG	Isopropylthio-β-D-galactoside
IS	Inverse PCR product of *Sau*3A-digested DNA
IVS	Intervening sequences
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
Pre-tRNA	Transfer ribonucleic acid precursor transcript
RNase P	Ribonuclease P
RF	Replication form
RT	Room temperature
SDS	Sodium dodecyl sulfate
TFIIIA, B, or C	Transcription factor III A, B, or C
Tris	Tris (hydroxymethyl) amino-methane
tRNA	Transfer RNA
X-GAL	5-Bromo-4-chloro-3-indolyl-β-D-galactoside
Ψ	Pseudouridine
ψ^+	A yeast extrachromosomal factor

INTRODUCTION

In recent years, considerable progress has been made in our understanding of transfer RNA and transfer RNA gene structure and function in prokaryotes and eukaryotes. Transfer RNAs are an essential part of the machinery involved in translating mRNA into protein on the ribosome. Transfer RNAs are small molecules within the cytoplasm, which carry different amino acids attached to their 3' end to the ribosome-mRNA complex, and which release the amino acid where the corresponding codon is met. In other words, they play the role of the "adaptor" molecule first proposed by Crick in 1955 (described in Zachau 1978).

The focus of this thesis is the function of a $tRNA^{Trp}$ gene in the yeast, *Saccharomyces cerevisiae*, as an ochre suppressor. It also is concerned with sequencing the flanking regions of different $tRNA^{Trp}$ genes. This introduction will review some of history of nonsense suppressors, as well as tRNA gene transcription and the involvement of the flanking regions.

Of the 64 possible codons in the triplet genetic code, 61 are used to code for the 20 amino acids and the other three (UAA, UAG, UGA) usually act as terminator codons. Exceptions to this rule occur in the genetic code of mitochondria of *Saccharomyces cerevisiae* and some other organisms, where, for example, UGA is read as tryptophan (reviewed in Fox 1987). A mutation within a messenger RNA that causes the conversion of an amino acid specifying codon into one of the three terminator codons leads to premature termination of

translation (also called "nonsense" mutation). The effect of such a mutation can be reversed by secondary extragenic suppressor mutations, or nonsense suppression. These are classified according to the nonsense codons on which they act: amber suppressors read UAG, ochre suppressors read UAA, and opal suppressors read UGA. In *E. coli*, ochre suppressors also read UAG (reviewed in Sherman 1982).

Benzer and Champe (1962) first recognized that nonsense suppression can be mediated by an altered tRNA. During studies of the genetic map of the rII region of bacteriophage T4, Benzer examined a number of rII mutants. Some of the rII mutants exhibited an ambivalent phenotype; that is, the mutant phenotype did not appear when the phage was grown on certain bacterial hosts. Benzer recognized that the ambivalence could be due to heritable differences in the decoding properties among several bacterial strains. In fact, he showed that some of the mutations in the rII region of the T4 are second site mutations which could be in a tRNA gene. Since then many cases of nonsense suppression have been shown to be mediated by tRNA (reviewed in Sherman 1982). For some of these, the locus of the suppressor mutation has been traced directly to the structural gene for a tRNA. This revealed that nonsense suppression generally results from a mutation in a "sense" anticodon to give the tRNA the ability to interact with a termination codon. However, a mutation in the anticodon is not the only way to generate a nonsense suppressor tRNA. For example, the *E. coli* UGA suppressor $tRNA^{Trp}$ differs from the wild type tRNA by a base change in the stem of the D-loop (Hirsh 1971).

Other studies, dealing with *Saccharomyces cerevisiae*, supported the arguement that suppression is mediated by mutationally altered tRNA molecules. *S. cerevisiae* strains containing UAG suppressors like *SUP6-a, SUP-RL1-a* (Gesteland et al. 1976), or UAA suppressors like *SUP4-o* (Gesteland et al. 1976), were shown to contain tRNA molecules capable of translating either UAG or UAA codons, respectively, in QβRNA and mammalian globin mRNA with *in vitro* protein synthesizing systems. DNA sequencing of the *SUP4-o* gene encoding the suppressor tRNA revealed a base pair change at the anticodon (Goodman et al. 1977). As in other organisms, altered yeast tRNAs with suppressor activity can have base changes within or outside anticodons, or they can have bases lacking proper modifications (Sherman 1982).

Numerous nonsense suppressors in *Saccharomyces cerevisiae* have been isolated and genetically mapped (reviewed in Hawthorne and Leupold 1974; and Sherman 1982). The action of many of these suppressors was determined using UAA or UAG alleles of *cyc1* which encodes iso-1-cytochrome C. In addition, the amino acid which was inserted by the suppressor was determined by sequencing the N-terminal region of the purified mutant protein. This type of study on UAG suppressors revealed that eight loci (*SUP2-SUP8* and *SUP11*) insert tyrosine at the nonsense codon (Liebman et al. 1976), or that *SUP61* yields a recessive lethal tRNA which insert serine (Etcheverry et al. 1982). Liebman et al. (1976) isolated dominant amber suppressors, classified them and showed that the most efficient suppressors were those which insert tyrosine at the site of nonsense codons. Liebman et al. (1984) isolated a group of amber suppressors

that were distinguishable from those which insert tyrosine by their suppression efficiency on the amber allele of *cyc1-179*, and mapped them by conventional genetic techniques. It was shown that all of these weak suppressors were loci coding for tRNAs which insert leucine. Also it was demonstrated that the context in which the mutation is located and the actual amino acid inserted influence the efficiency of suppression.

While genetic aspects of nonsense suppressors in yeast were under investigation, modifiers of nonsense suppression were also found. These were chromosomal or extrachromosomal factors which influenced, enhanced or reduced, the efficiency of suppression by certain nonsense suppressors. The mutations that reduce the efficiency of suppression are referred to as antisuppressors. In contrast mutations enhancing the efficiency of suppression have been referred to as allosuppressors. Numerous antisuppressors have been identified (McCready and Cox 1973, Laten et al. 1978, Liebman and Sherman 1976, Hawthorne and Leupold 1974, and Gorman and Gorman 1971) but the primary defect of only the *mod5* antisuppressor has been identified; tRNAs from the *mod5* antisuppressor contain only 1.5% of the normal level of isopentenyladenine (Laten et al. 1978). The *MOD5* gene was cloned by screening ura$^+$ transformants of a *SUP7 mod5-1* strain for restoration of suppression of *ade2-1* (Diahanich et al. 1987). *MOD5* is suggested to be the gene that codes for Δ^2-isopentenyl pyrophosphate-tRNA isopentenyl transferase (IPP Transferase) (Najarian et al. 1987 and Diahanich et al. 1987). Recessive mutations

of five distinct loci have been shown to give rise to the allosuppressors *Sal1-Sal5*, which increase efficiency of suppression of ochre nonsense codons by *SUP16* (Cox 1977). Another group of sal allosuppressors, which may be different than *sal1-sal5*, increases the suppression efficiency of the weak UAA suppressor *SUP20* (or *SUP19*) and the mutated UAG suppressor *SUP5* when present with the antisuppressor *SIN2* (Hawthorne and Leupold 1974).

In addition to chromosomal determinants which modify the pattern of suppression, a non-Mendelian genetic determinant, ψ^+, was identified by its ability to enhance the expression of an ochre suppressor, *SUQ5*, (Cox 1965, 1971). ψ^+ was discovered to be an extrachromosomal determinant using a red adenine-requiring revertant of a strain in which the *ade2-1* mutant allele was suppressed by the nonsense suppressor *SUQ5*. When the revertant was crossed to a parental strain (*SUQ5, ade2-1*), all of the products of meiosis were white and adenine independent. The red adenine-requiring mutant phenotype failed to segregate, suggesting that an extrchromosomal determinant existed in yeast, which in one form enhanced the nonsense suppressing activity of the *SUQ5* suppressor (a serine-inserting tRNA). The ability of the ψ^+ factor to enhance suppression also holds true for other ochre suppressors such as *SUP17* or *SUP19* (Ono et al. 1979). We present evidence in this thesis that ψ^+ also enhances suppression of an ochre mutation by a plasmid-borne intronless ochre suppressor $tRNA^{Trp}$. Combination of ψ^+ with strong ochre suppressors like *SUP11* (which is formed by mutation of the anticodon of $tRNA^{Tyr}_{AUA}$ to read UAA and is a dominant strong suppressor in a ψ^- background) causes lethality.

Similarily, *SUP2-8* are dominant lethals in a ψ+ strain. To date, no amber or opal suppressor has been found whose *in vivo* phenotypic effects have been modified by ψ+. The amber allele of *SUQ5* is unaffected by ψ+. However, Cummins et al. (1980) showed that the level of suppression due to frameshift suppressors *SUF1*, *SUF4*, and *SUF6* was increased in ψ+ strains compared to ψ−. The action of the ψ+ factor is eliminated in a hypertonic medium containing KCl or ethylene glycol (Singh et al. 1979). It is known that the ochre mutation *trp5-48* is suppressed in a ψ+ background in the absence of ochre suppressors (reviewed in Cox et al. 1988). Also, Liebman and Sherman (1979) found that the ochre mutation *cyc1-72* yielded about 1% of wild type levels of iso-1-cytochrome C in ψ+ strains, but undetectable levels in ψ− strains. These data suggest that the ψ+ factor alone has a weak ochre suppressor ability. The precise intracellular location of the ψ+ factor and the way it increases the efficiency of ochre and certain frameshift suppressors is still unknown. Attempts at mapping the ψ+ behavior to known extrachromosomal nucleic acids in yeast (mitochondrial DNA, 2μm ds DNA circular plasmid, and the linear dsRNA associated with the killer character) have failed (Tuite et al. 1982, Young and Cox 1972). Dai et al. (1986) transformed ψ− strains of yeast to ψ+ using different fractions of DNA extracted from a ψ+ strain. They showed that the transforming activity co-purified with a 3μm plasmid encoding rRNA prepared from ψ+ strains. Southern blotting and probing with rDNA reveals that 3μm DNA is present both in ψ+ and ψ− strains (Dai H. unpublished observation) suggesting that if ψ+ function is provided by 3μm circle, then some mutations may alter function in ψ− strains.

Furthermore, if the 3μm circle, indeed, codes for ψ^+, it does not make it any easier to understand ψ^+ function in enhancing the activity of nonsense suppressors. Different models have been suggested for the action of ψ^+, however, none of them is compelling (reviewed in Cox et al. 1988)

Another important aspect of tRNA genes is their organization, transcription, and processing. Transfer RNAs in both prokaryotes and eukaryotes are encoded by small genes dispersed around the genome. In *E. coli*, 54 tRNA genes, encoding 35 subspecies, representing an estimated two-thirds of the total number of tRNA genes, have been mapped (reviewed in Fournier and Ozeki 1985). Of the 54 tRNA genes, 37 occur in polycistronic operons containing other tRNA or rRNA genes. All *E. coli* tRNA genes encode the 3'-CCA terminus typical of all tRNAs. In eukaryotes, tRNAs are usually encoded by multicopy genes but the organization of these genes varies from species to species (Sharp et al. 1985). In *Drosophila melanogaster*, the estimated number of tRNA genes ranges from 590 to 750 (Rittosa et al. 1966, Weber and Berger 1976). The tRNA genes of Drosophila are typically clustered in several regions (Robinson and Davidson 1981). There are approximately 360 tRNA genes in *Saccharomyces cerevisiae* and 200 to 300 in *Schizosaccharomyces pombe* (Schweizer et al. 1969; Feldmann 1976) as judged by DNA hybridization. On average there are eight genes per tRNA species. The only juxtaposition of tRNA genes in *Saccharomyces cerevisiae* reported to date was by Beckmann et al. (1977) suggesting that there is little clustering of tRNA genes. They screened a yeast genomic DNA library of 4000 clones containing random fragments. About 175 of

these were shown to contain tRNA genes. Out of 175 clones, 4 contained genes for $tRNA^{Arg}_3$ and $tRNA^{Asp}$. DNA sequencing of these two tRNA genes finally showed that they are tightly linked (Schmidt et al. 1980). Similarily, in *Schizosaccharomyces pombe*. sequencing analysis of DNA fragments revealed a $tRNA^{Ser}_{UCG}$ gene at the 5' side of an initiator $tRNA^{Met}$ gene (Jen-Mao et al. 1980) separated by a seven nucleotide spacer. It has also been shown that centromere associated B' and B DNA sequence repeats of *S. pombe* chromosomes I and II contain clusters of tRNA genes. The centromere of chromosome II (CEN2) includes at least 22 tRNA genes and the CEN1 region contains at least six tRNA genes (Kuhn et al. 1991). The reason for this unusual positioning is still unknown.

Some of the tRNA genes code for isoaccepting tRNAs. Among these, the best studied in *S. cerevisiae* are those for $tRNA^{Tyr}$. This tRNA is transcribed from eight different genes. Estimates based on the amount of suppressor anticodon relative to the wild type sequence are consistent with roughly equivalent expression of seven of these eight genes (e.g. Colby et al. 1981). However, the level of suppression in the *SUP11* strain is markedly lower than that of the other seven suppressors, which brings up the possibility that this tRNA is transcribed and/or processed at a lower efficiency. Similarily, the three $tRNA^{Ser}_{UCA}$ genes are expressed at different levels, even though the genes encode identical tRNAs (Broach et al. 1981).

About 10 to 20 percent of nuclear encoded tRNA genes in eukaryotes contain introns (reviewed in Sharp et al. 1985). These introns range from 8 to 60 nucleotides in length and are homologous within any family, but are divergent among different gene families.

It was first shown by Goodman et al. (1977) that the eight $tRNA^{Tyr}$ coding regions were interrupted by additional nucleotides. Olson et al. (1981) demonstrated that yeast serine-inserting suppressors, $tRNA^{Ser}_{minor}$ species, are transcribed from a family of four genes and two of these have been identified with the genetic loci *SUP-RL1* and *SUQ5*. They showed that although these two suppressor genes differ by only three bp within their coding regions, the *SUP-RL1* gene has an intervening sequence whereas the *SUQ5* gene does not. Clear evidence for the presence of introns in several tRNA genes of yeast was obtained from the characterization of a set of tRNA precursors accumulated in a mutant strain (Hopper et al. 1978). They found that a temperature sensitive mutant of yeast known as *rna1* (ts 136) accumulates tRNA precursors. The mutant is presumed to be defective in the transport of RNA from nucleus to cytoplasm at the nonpermissive temperature. Direct evidence was provided by separating RNA molecules on two dimensional polyacrylamide gels, followed by hybridizing with recombinant plasmids encoding different tRNAs and sequencing (Knapp et al. 1978, O'Farrell et al. 1978, Kang et al.1980). The results indicated that there are at least 9 sets of tRNA genes that contain intervening sequences. Many other specific tRNA genes have been found to contain introns. For example, the six members of the $tRNA^{Trp}$ gene family in yeast, the subject of this study, have identical coding sequences as well as identical 34 bp introns (Atkin et al. 1992a).

In eukaryotic cells, RNA polymerase III is responsible for the transcription of 5S RNA, tRNA, some cytoplasmic and nuclear small RNAs, and some virus-associated RNAs (reviewed in Geiduscheck and

Tocchini-Valentini 1988). It has been known for more than a decade that the promoter elements of the genes transcribed by RNA polymerase III are located within the coding regions (Sakonju et al. 1980, Sharp et al. 1981, and Ciliberto et al. 1982a). The internal promoter of tRNA genes is split into two essential regions of about 10 nucleotides each, which are highly conserved. These two regions were referred to as "box A" or 5' ICR (internal control region) and "box B" or the 3' ICR. The distance between these two regions ranges from 30 to 74 bp, depending on the presence of an intron and the size of the variable loop. This distance was defined by functional analysis of deletion and substitution mutants of a variety of tRNA genes (Galli et al. 1981; Ciliberto et al. 1982a,1982b,1983).

In addition to the ICRs, it was first shown in a homologous cell-free system (*in vitro* transcription) that the 5' flank of some tRNA genes markedly affects the efficiency of transcription (Defranco et al. 1981; Dingermann et al. 1982). The 5' and 3' boundaries of regions involved in transcription were found to differ between tRNAs and organisms. Using a deletion analysis, Defranco et al. (1981) demonstrated an inhibitory consensus sequence 5' to the $tRNA^{Lys}_2$ genes in Drosophila, which was located 25 base pairs upstream of the transcription start site. On the other hand, *in vitro* mutagenesis and deletion studies showed that a positive modulatory sequence is present between positions -34 to -38 of the $tRNA^{Val}_4$ gene of Drosophila (Sajjadi and Spiegelman 1989). The effects of altered 5' flanking sequences on the *in vivo* expression of *S. cerevisiae* $tRNA^{Tyr}$ genes was examined by constructing nested deletions ending in the 5' flanking sequences of the *SUP4-o* gene, and then testing the

suppressor phenotype of the relevant constructs after transformation into *S. cerevisiae* (Shaw and Olson 1984). This study showed that deletions which retain only 4 base pairs of the 5' flanking sequence are profoundly deficient in expression; deletion mutants extending into approximately -16 (from the first base of tRNA) were moderately deficient; and deletions into -27 were only slightly defective. Similar deletion studies on *SUP4-o* 3' flanking regions demonstrated that deletions extending into the T cluster in the 3' flanking sequence decrease the ability of *SUP4-o* to compete for a transcription factor. Therefore, the sequences in the 3' flank are not only involved in termination, but also somehow involved in transcription (Allison and Hall 1985). Using primer extension analysis for a $tRNA^{Val}$ gene in *S. cerevisiae,* containing different small linkers inserted in its 5' noncoding regions, Marschalek and Dingermann (1988) demonstrated that a few essential nucleotides within the flanking region are able to determine the *in vivo* transcription activity of this tRNA gene. Since no consensus sequence has been found among different tRNA genes even though the ICRs are well conserved, the differences in transcription efficiencies could be attributed to differences within the 5' flanking regions (Marschalek and Dingermann 1988). Also, it has been found that replacing the 5' flank of a tRNA gene in yeast with a random stretch of DNA does not permit transcription (Straby 1988), in contrast to HeLa cell or Xenopus oocyte systems where any stretch of DNA can function efficiently as a 5' flank for tDNA (Ciliberto et al. 1984).

How do flanking sequences act in and influence the transcription complex? The recognition of the ICRs by RNA polymerase III is

mediated by transcription factors specific to the internal promoter regions, TFIIIB and TFIIIC. 5S RNA gene transcription requires an additional factor TFIIIA (reviewed in Murphy et al. 1989). It was hypothesized by Dingermann et al. (1983) that TFIIIB and TFIIIC are the factors necessary for transcription. However, there might be other protein factors which bind to 5' or 3' flanking sequences and are involved in the stability of the transcription complex. It was later shown that TFIIIC binds to the A and B boxes but is not sufficient to activate specific transcription by RNA polymerase III (Klekamp and Weil 1986, and Camier et al. 1985). The other required transcription factor TFIIIB is sequestered into stable tRNA gene transcription complexes only in the presence of TFIIIC, suggesting that protein-protein interaction is essential for further complex assembly (Lassar t al. 1983). Other studies demonstrated an upstream binding activity which copurifies with TFIIIB. *SUP4* gene DNA that was cut within the 5' flanking sequence (either 31 or 28 bp upstream of the transcriptional start site) was no longer able to stably incorporate TFIIIB into a transcriptional complex (Kassavetis et al. 1989).

Genomic footprinting showed that the 5' flanking region of the *S. cerevisiae* tRNA *SUP53* gene is protected from DNaseI digestion (Huibregtse and Engelke 1989). The protection of the upstream region could be due to binding of one or more distinct transcription factors, perhaps including TFIIIB. The order of assembly of transcription complexes on the *SUP4* $tRNA^{Tyr}$ gene was examined by measuring the sensitivity of the DNA sequences encoding the tRNA genes to DNaseI, following the addition of different purified transcription factors. The assay demonstrated that TFIIIC protects

boxB and partially protects boxA of the internal promoter from cleavage by DNaseI. Adding TFIIIB strengthens the footprint attributed to TFIIIC, but enhances DNaseI cleavage of the transcription initiation site and extends protection 42 base pairs upstream of the transcription start site. Addition of RNA polymerase III generates protection in the region surrounding the transcription start site.

Further studies suggested that once TFIIIC has positioned TFIIIB upstream of the transcription start site of the tRNA genes, it is dispensable for transcription by RNA polymerase III. In other words, TFIIIB is the sole transcription initiation factor of *S. cerevisiae* RNA polymerase III, and TFIIIC is an assembly factor for TFIIIB (Kassavetis et al. 1989, 1990).

Recently it has been shown that yeast TATA binding protein is essential for the transcription of the genes transcribed by RNA polymerases I, II, and III (Cormack and Struhl 1992) using $tRNA^{Trp}$ and $tRNA^{Leu}$ genes and showing that elimination of yeast TBP (TATA binding protein) significantly affected transcription. They proposed that TBP can possibly bind to the TATA sequence commonly found at the upstream region of tRNA genes in yeast.

Another interesting observation related to this subject is the targetted insertion of Ty3 elements upstream to tRNA transcription start sites. This usually happens within 16 or 17 nucleotides from the 5' ends of coding region for mature tRNAs, even though no insertion consensus sequence could be found and there is no similarity between Ty3 and tRNA gene sequences (Chalker and Sandmeyer 1990). Recently, Chalker and Sandmeyer (1992) cotransformed yeast

with two different plasmids, one in low copy number and containing a *URA3A* gene as a selectable marker and a Ty3 element tagged to a *neo*^{R} gene, and the other plasmid in high copy number and having a *SUP2* gene as a target for Ty3 insertion and a *HIS3* selectable gene. G418 resistant cells were selected which were able to grow on 5-FOA-containing media as well as on his^{-} plates. In other words, they had lost the ura^{+} carrying plasmid but not the plasmid having the his^{+} gene. DNA sequence analysis showed that Ty3 elements were exclusively inserted upstream of the plasmid-borne *SUP2* tRNA gene. When the transcription of the tRNA gene was impaired by a mutation in boxB, thus preventing TFIIIC binding, no targetting happened. Rather, integrations were random. Finally, they proposed that it is not a specific sequence in the upstream region of tRNA genes but the polymerase III transcription factors which cause the targetted insertion of Ty3.

Maturation of tRNA molecules, including the splicing of introns, is relatively well understood (reviewed in Atkin 1992). The maturation of eukaryotic pre-tRNA molecules include the removal of 5' leader and 3' trailer sequences, addition of the 3' -CCA tail, splicing of any intervening sequences, nucleotide modifications and transport from the nucleus to the cytoplasm. Both genetic and biochemical methods have been used to study tRNA processing. By using conditional loss of function as an assay, Hopper et al. (1980) screened for a yeast mutant, *los1-1*, which is defective in tRNA processing. Like *rna1*, tRNAs with intervening sequences seem to accumulate in *los1-1* cells. Both *los1-1* and *rna1* have been shown to have normal tRNA splicing

activities *in vitro* (Hurt et al. 1987, and Winey and Culbertson 1988). Therefore, probably they do not play a direct role in the enzymology of tRNA intron removal. In addition to modification-deficiency mutations, 12 *S. cerevisiae* genes have been identified that affect tRNA processing (O'Conner and Peebles 1991).

On the basis of Xenopus oocyte microinjection studies of cloned *S. cerevisae* tRNA genes (DeRobertis et al. 1981, and Melton et al. 1980) and *in vitro* experiments with *S. cerevisiae* and Xenopus partially and purified enzymes (Ogden et al. 1979, and Engelke et al. 1985), a generally accepted pathway for tRNA processing has been obtained. The first reaction appears to be the endonucleolytic removal of the 5' leader by RNase P. RNase P (from both prokaryotic and eukaryotic sources) is an endonuclease containing RNA and protein. In prokaryotes both RNA and protein components of the ribonucleoprotein enzyme are required for *in vivo* activity. However, the RNA alone is capable of removing the 5' leader *in vitro* under some conditions (Jain et al. 1982). In eukaryotes, the identification of the RNA as the essential component for RNase P activity has not been demonstrated (Lee et al. 1991). Precursor tRNAs having both 5' leader and 3' trailer do not appear to be substrates for the Xenopus 3' end trimming activity (Castano et al. 1985), therefore the 3' trailer must be removed after the 5' leader is trimmed. It is not known whether the 3' end trimming activity is an endonuclease, an exonuclease, or a combination of both (Castano et al. 1985). The $-CCA_{OH}$ is then added to the 3' end by a nucleotidyltransferase (Deutscher 1970). The end-matured unspliced tRNA was believed to be the precursor for splicing. However, it is known that splicing of

the intron can happen before or after end maturation (O'Conner and Peebles 1991). The first step for intron removal is an ATP-independent endonucleolytic incision. *In vitro*, intron-containing pre-tRNA is cut first at the 3' splice site and then at the 5' splice site (Reyes and Abelson 1988). The reaction generates 2',3' cyclic phosphates and 5' hydroxyl groups. Then tRNA ligase forms a new phosphodiester bond between the two tRNA exons in an ATP-dependent reaction that also leaves a 2' phosphate at the junction. The 2' phosphate is subsequently removed by what appears to be an NAD^+-dependent phosphatase (McCraith and Phizicky 1990).

The function of introns in tRNA genes is not understood. Various studies have shown different results, both *in vivo* and *in vitro*. Sequence analysis of several split tRNA genes from yeast has revealed several characteristics of the introns. They reside one base to the 3' side of the anticodon. Within a tRNA gene family their sequences are identical, or nearly so, but there are no similarities between families. Finally, the introns always contain a sequence complementary to the anticodon. Pairing between these sequences may be involved in the secondary structure of the precursor. Johnson and Abelson (1983) reported the construction of a mutant $tRNA^{Tyr}$ ochre suppresssor gene (*SUP6Δ32*) from which the 14 bp IVS had been deleted. *SUP6* or *SUP6Δ32* were then integrated into a yeast strain carrying different suppressible ochre mutations. It was shown that removal of the IVS converts the *SUP6* gene to a less efficient suppressor. When *in vivo* labeled $tRNA^{Tyr}$ from the two strains (*SUP6* and *SUP6Δ32*) was examined, *SUP6*-specific tRNA from the deletion strain lacked a specific pseudouridine (Ψ). They proposed

that the failure to make this modification is responsible for the decrease in suppressor efficiency of the mutant. Another study on *SUP53* (a leucine-inserting amber suppressor) showed similar results (Strobel and Abelson, 1986a). Different *SUP53* constructs which carried either a deletion of the 5' or 3' side of the intron or a complete intron deletion were transcribed *in vitro* using a yeast nuclear extract. Most mutants were transcribed and end-processed efficiently, however, ΔIVS and Δ3'IVS primary transcripts were not. When the *in vivo* labeled $tRNA^{Leu}_s$ were compared, only the *SUP53* tRNA contained the expected 5-methylcytosine modification. In addition, the *in vivo* suppression assay showed that the ΔIVS *SUP53* was not as efficient a suppressor as *SUP53* (Strobel and Abelson 1986a). Studies on modification of U to Ψ proposed that general features of the tertiary structure of the intron-containing pre-tRNA molecules are the major determinants for the recognition by the enzyme responsible for modification, rather than specific sequences of the anticodon loop or intron (Choffat et al. 1988).

Other studies have demonstrated the involvement of introns in their own removal. Raymond and Johnson (1983) made a partial deletion of a *S. cerevisiae* $tRNA^{Leu}_3$ gene intron *in vitro*. In a cell extract of *S. cerevisiae* this deletion mutant was transcribed efficiently and end-processed but was not spliced. In another study the splicing activity of several intron mutation-containing *SUP53* genes was examined and the *in vitro* phenotype of these mutants were correlated with their tRNA suppressor function *in vivo* by integration of the mutant genes into the yeast genome (Strobel and Abelson 1986b). It was demonstrated that all poorly spliced or

unspliced precursors were blocked in the production of 3' half molecules. The precursors which produced mature tRNA had substantial single stranded regions at the 3' splice junction. This and other studies (Kang et al. 1979, Odgen et al. 1984, Atkin et al. 1992b, and this study) demonstrate that base pairing between intervening sequences and the anticodon loop at the 3' splice junction has a critical role in splicing. This is also supported by study of *SUF7* and *SUF8* (the proline inserting +1 frameshift suppressors). *In vitro* experiments demonstrated that the splicing efficiency is reduced in these mutants compared to the wild type $tRNA^{Pro}$ (Winey et al. 1986). It was proposed that this reduced splicing efficiency resulted from inefficient endonuclease cleavage at the 3' splice junction, presumably because of an inappropriate secondary structure of the tRNA for the endonuclease to cleave this site. Deletion of the intron from the gene (*SUF8*) by *in vitro* manipulation resulted in enhanced efficiency of suppression, supporting the notion that the *SUF8* pre-tRNA has difficulty in splicing (Winey et al. 1986). The same study also demonstrated that tRNA produced from the intron-deleted *SUF8* gene is identical to that of the product of the intact gene, indicating that the intron is not required for proper base modification.

Further investigations on *SUP61-a* and *SUP61-o* (serine-inserting tRNAs suppressing amber and ochre alleles, respectively) and the corresponding intronless genes showed that the *sup61*$^+$ intron is not essential for either transcription or suppression, as there was no significant difference in the level of mature $tRNA^{Ser}_{UCG}$ produced *in vivo*, and also no difference in the efficiency of suppression by *SUP61* or *SUP61-ΔIVS* genes. Overall, there is no general role for

intervening sequences in tRNA genes. This study and Atkin et al. (1992b) present some evidence suggesting that the intron of a $tRNA^{Trp}$ gene is not passive, but that certain bases are critical for the appropriate tRNA secondary structure for the endonuclease enzyme to cleave the intron.

The end-matured, spliced tRNA is transported from the nucleus to the cytoplasm, either during or after splicing (Melton et al. 1980), where it interacts with the corresponding aminoacyl synthetase enzyme to be acylated with an amino acid. Each amino acid is usually represented by more than one cognate tRNA in the cell. Isoaccepting tRNAs are a group of tRNAs that have the same amino acid specificity and are acylated with the cognate amino acid by a single aminoacyl tRNA synthetase. Therefore, they should contain the same identity elements for tRNA synthetase (Schulman and Abelson 1988). Both biochemical and genetic studies have indicated that the anticodon plays a major role in defining the amino acid that will be attached to many tRNAs (reviewed in Kisselev 1983, Normanly and Abelson 1989). Genetic experiments have shown that conversion of $tRNA^{Arg}$ I(ICG) into an amber suppressor tRNA having the anticodon CUA results in partial loss of arginine acceptor acivity and insertion of mostly lysine into protein at the site of amber codons (McClain and Foss 1988).

Using recombinant RNA technology, Bruce and Uhlenbeck (1982) have demonstrated that substitution of any of the yeast $tRNA^{Phe}$ anticodon nucleotides decreases specific aminoacylation 3 to 10 fold. In addition, transplanting the phenylalanine anticodon onto $tRNA^{Tyr}$ leads to aminoacylation of the hybrid molecule by the phenylalanine

AAS (Bare and Uhlenbeck 1985). In this experiment the aminoacylation of the hybrid tRNA was fairly weak, suggesting that there must be phenylalanine recognition elements outside of the anticodon as well. For *E. coli* $tRNA^{Ala}$, it was shown that the anticodon is not an essential part of the identity set (Hou and Schimmel 1988; McClain and Foss 1988). By site specific mutational studies, it was demonstrated that substitution of the G-U base pair in the acceptor stem eliminated aminoacylation with alanine *in vivo* and *in vitro*.

It has also been shown in one case that the accuracy of *in vivo* aminoacylation requires proper balance of tRNA and aminoacyl-tRNA synthetase. When *E. coli* Gln-tRNA synthetase was overproduced *in vivo*, it incorrectly acylates the *supF* amber suppressor $tRNA^{Tyr}$ with glutamic acid. The effect was abolished when the intracellular concentration of the cognate $tRNA^{Gln}_2$ is also elevated (Swanson et al. 1988). A recent study by Graffe et al. (1992) showed that the threonyl-tRNA synthetase binds to the region of the cognate mRNA leader that has structural analogies with the natural substrate ($tRNA^{Thr}$), when the concentration of cognate tRNA is low. In this way the enzyme negatively autoregulates itself.

PRESENT INVESTIGATION

The *in vitro* construction of a functional $tRNA^{Trp}$ suppressor in *S. cerevisiae* was first demonstrated by Kim and Johnson (1988), wherein they created a functional amber suppressor by changing a base in the anticodon. However, it was subsequently shown that an *in vitro* constructed opal suppressor $tRNA^{Trp}$ was a weak suppressor *in vivo*. Atkin et al. (1990) suggested that an alteration in the

secondary structure of the opal suppressor $tRNA^{Trp}$ leave the 3' splice junction double stranded, which inhibits the recognition and/or cleavage by the tRNA splicing endonuclease. Second site mutations in the intervening sequences of the gene restored the function of the splicing enzyme and made a stronger suppressor. In subsequent studies (this study, and Atkin et al. 1992b), it was shown that an ochre suppressor $tRNA^{Trp}$ was not functional even after introducing second site mutations which hypothetically encode a transcript which should leave the 3' splice junction single stranded. Northern hybridization studies showed very little mature ochre suppressor $tRNA^{Trp}$ was present in the bulk tRNA extracted from cells transformed with these constructs.

In the present study, this hypothesis was tested by deleting the intron from a putative ochre suppressor $tRNA^{Trp}$ gene. The intronless $tRNA^{Trp}$ gene then was subcloned either into a low copy number yeast-*E. coli* shuttle vector, YCp50, or into high copy number vectors, YRp17 and/or YEp352. Different ψ^+ or ψ^- yeast strains were transformed with these constructs and the *in vivo* suppression patterns were examined. The intronless suppressor $tRNA^{Trp}$ gene showed a relatively stronger suppression of different ochre mutations, compared to the cognate ochre suppressor $tRNA^{Trp}$ genes with introns. In addition, the ψ^+ factor was demonstrated to be effective in increasing the efficiency of suppression.

In the other part of the present work, attempts were made to amplify and sequence 5' and 3' flanking regions of the different $tRNA^{Trp}$ genes in *S. cerevisiae*. It has been shown that there are six different $tRNA^{Trp}$ genes present in *S. cerevisiae* (Atkin et al. 1992a).

The 5' and 3' flanking sequences of only one of these genes has been published (Kang et al. 1980). In this study an inverse PCR technique was used to amplify the flanking regions of the six genes. Using this technique, I was able to amplify the flanks of five of these genes. Finally, three of the fragments were sequenced.

MATERIALS AND METHODS

The bacterial and yeast strains as well as the cloning vectors utilized in this thesis are listed and briefly described in table 1.

Preparation of *E.coli* Competent cells and Transformation

Bacterial strain, DH5α, was grown overnight in LB medium (Sambrook et al. 1989) at 37°C. A 0.5 ml aliquot of this was used to inoculate 19.5 ml LB, and grown at 37°C until it reached log phase of growth. The flask was placed on ice for 30 minutes, then the cells were centrifuged at 5000 rpm for 10 min., resuspended in a half volume of cold 50 mM $CaCl_2$ and incubated on ice for 30 minutes. Finally, the cells were pelleted and resuspended in one tenth volume of cold 50 mM $CaCl_2$ and stored at 4°C. These cells could be used up to 48 hrs later. A 120 µl aliquot of these cells were transferred to a 1.5 ml Eppendorf tube and 1 µg of DNA was added, kept on ice for 40 min, and then heat shocked at 42°C for 90 seconds, followed by the addition of 880 µl of LB, and the tubes were incubated at 37°C for 30 min with shaking. Finally, 100-200 µl was plated on either LB or McConkey agar containing 60 µg/ml of ampicillin. For transformation with M13 DNA, 150 µl of an F' strain, JM105 or XL1-Blue , was added to the heat-shocked cells, then 5 µl of IPTG (25 mg/ml) and 50 µl of X-Gal (25 mg/ml) were added, mixed and the contents were poured onto a LB plate. The white plaques, M13s carrying the insert, were picked up for further experiments.

Table 1: The bacterial and yeast strains as well as the cloning vectors used in this study

Name	Genotype	Use	Source
Bacteria *(Escherichia coli)*			
DH5α	*supE44 lacU169(ϕ80 lacZΔM15) hsdR17 rec1 gyrA96 thi1 relA1*	transformation and cloning of the tRNA genes	Hanahan 1983
JM105	*thi1 rpsL endA sbcBC hspR4 (lac-proAB)[F' tra D36 proAB lacIqZΔM15)*	host for M13 phage vectors or their derivatives	Yanisch-Perron et al. 1985
BW313	*Hfr KL16 Pol45 (LysA(61-62)) thi1 relA1 spoT1 dut1 ung1*	host in preparing ssDNA containing deoxyuridine	Tye et al. 1978
XL1-Blue	*supE44 hsd R17 recA1 end A1 gyr A46 relA1 lac⁻ F' [pro AB⁺ lacIqlacZΔM15 Tn10(tet^r)]*	host for M13 phage vectors and their derivatives	Levinson et al. 1984
Yeast *(Saccharomyces cerevisiae)*			
JG369-3B	*MATα ade2-1* (UAA) *lys2-1* (UAA) *can1-100* (UAA) *trp1-1* (UAG) *met8-1* (UAG) *leu2-2* (UGA) *his4-261* (UGA) *ura3-52*	*in vivo* suppression assay	J.G. Gelugne
JG113-5R	*MATα ade2-1 can1-100 trp1-1 met8-1 leu2-2 his4-260*	DNA isolation and inverse PCR	J.G. Gelugne
RVB-4B	*MATa trp5-48 ura3-52 ade2-1 lys1-1 hom3-10 leu2-3,112 his1-7*	*in vivo* suppression assay	R.C. von Borstel

RVB-45C	*MATa his3-11,15 leu2-3,112 lys1-1 ade2-1 trp1-1 ura3-52* ψ^+	*in vivo* suppression assay	E. Savage
AB1380	*MATa ade2-1 can1-100 lys2-1 trp1-1 ura3 his5* ψ^+	*in vivo* suppression assay	F. Nargang
XV185-6A	*MATα ade2-1 arg4-17 his1-7 lys1-1 trp5-48*	testing for the presence of ψ^+ factor in RVB-45C	E. Savage
YO600-7C	*MATα ade2-1 arg4-17 hom3-10 his1-7 lys1-1 trp5-48*	testing for the presence of ψ^+ factor in RVB-45C	P. Hastings
YO600-1C	*MATα ade2-1 his1-7 lys1-1 hom3-10 arg4-17 trp5-48 met8-1*	testing for the presence of ψ^+ factor in RVB-45C	P. Hastings
Vectors			
pUC19	amp *lacZα*	used for cloning the $tRNA^{Trp}$ genes	Norrander et al. 1983
M13mp18,19	*lacZα*	preparation of single stranded DNA and sequencing	Norrander et al. 1983
YCp50	*ARS1 Ura* amp tet pyrF	subcloning of $tRNA^{Trp}$ genes and yeast transformations for *in vivo* suppression assay	Ma et al. 1987
YRp17	*ARS1 Ura* amp tet pyrF trpC	subcloning of $tRNA^{Trp}$ genes and yeast transformations for *in vivo* suppression assay	Botstein and Davis 1982
YEp352	2 μm ori *Ura* amp	subcloning of $tRNA^{Trp}$ genes and yeast transformations for *in vivo* suppression assay	Hill et al. 1986

Preparation of Yeast Competent cells and Transformation

In order to prepare competent yeast cells, 2 ml liquid YEPD (yeast complete medium, Rose et al. 1990) was inoculated with the cells, and grown overnight to 1-2 x 10^7 cells/ml. Then, the culture was diluted to 2 x 10^6 cells/ml in fresh, warm YEPD and regrown to 1 x 10^7 cells/ml. The cells were pelleted and washed in sterile water, resuspended in 1 ml sterile water, pelleted again, washed in 1 ml of freshly made 1 x TE (10 mM Tris.Cl, 1 mM EDTA) / LiAc (Lithium Acetate) and finally resuspended at 2 x 10^9 cells/ml in 1 x TE / LiAc. Competent yeast cells were used immediately after preparation. Fifty µl of the competent cells were transformed with 1-2 µg of DNA and 50 µg of single-stranded salmon sperm DNA in microfuge tubes. Then, 300 µl sterile 40% PEG 4000 solution (40% PEG 4000, 1 x TE, 1 x LiAc) was added and mixed, incubated at 30°C for 30 min, and then heat-shocked in 42°C for 15 minutes. The cells were spun down , resuspended in TE, and plated on ura$^-$ selective media.

Restriction Enzyme Digests and Ligation of Fragments

Digestion reactions were done in either 50 or 20 µl volumes using 10 units of the enzymes as in Sambrook et al. (1989). The enzymes and the buffers were purchased from BRL. Ligation reactions were also done in 30 µl using T4 DNA ligase and 5 x ligation buffer purchased from BRL.

Minipreparation of Plasmid DNA

Two protocols were used for the small-scale preparation of

plasmids:

1-The procedure of Chowdhury (1991) was used as follows: 0.5 ml of phenol:chloroform:isoamylalchohol (25:24:1) was added to an equal volume of an overnight culture of the transformants having the plasmid. This was vortexed for 1 min and then centrifuged at 14000 rpm for 5 minutes. The aqueous phase was added to 0.5 ml of isopropanol, mixed, and centrifuged immediately for 5 minutes. The pellet was washed twice with 70% ethanol and then dissolved in 100 µl of TE containing 20 µg/ml RNase.

2-The alkaline lysis method was used as described in Sambrook et al. (1989). A 1.5 ml aliquot of an overnight LB culture was pelleted and resuspended in 100 µl of ice-cold solution 1 (50 mM glucose, 10 mM EDTA, 25 mM Tris-Cl pH 8.0). Lysozyme was added to a concentration of 4 mg/ml. This was stored for 5 min at room temperature after which 200 µl of freshly-made solution 2 (0.2 N NaOH, 1% SDS) was added, mixed, and stored on ice for 5 minutes. Then, 150 µl of the ice-cold potassium acetate solution (3M potassium and 5M acetate) was added, stored on ice for 5 minutes, centrifuged for 5 min at 4°C, and the supernatant was transferred to a fresh tube. A phenol/chloroform extraction was done by adding an equal volume of phenol/chloroform, mixing, centrifuging for 3 min, and transferring the supernatant to a fresh tube. The nucleic acids were ethanol precipitated and washed once with 70% ethanol. The pellet was dried and dissolved in 50 µl of TE containing DNase free RNase (20 µg/ml).

Large-scale preparation of plasmid DNA

Two hundred ml of SBG medium was inoculated with 5 ml of an overnight culture of the transformed bacteria containing the plasmid. Ampicillin was added to a concentration of 60 µg/ml, when required. This was incubated at 37°C overnight, and the bacterial cells were harvested by centrifugation at 4000 rpm for 5 minutes. The cells were washed with ice-cold STE (0.1 M NaCl, 1 mM EDTA, 10 mM Tris-Cl pH 8.0) solution, pelleted again, and lysed in solution 1 (as described above), and then in 20 ml of solution 2 (2 M NaOH, 1% SDS). Then 15 ml of potassium acetate solution (3 M potassium, and 5 M acetate) was added, the preparation stored on ice for 10 min, centrifuged, the DNA precipitated with 0.6 volume of isopropanol, and pelleted by centrifugation at 5000 rpm for 15 minutes. The pellet was resuspended in 10 ml of TE.

Plasmid DNA was purified by cesium chloride-ethidium bromide gradient centrifugation. CsCl was added to a final concentration of 1 g/ml, and ethidium bromide was added to a concentration of 8 mg/ml. The preparation was then transferred to VTi65 rotor tubes and centrifuged at 54K for 12 hrs. The lower band (closed circular plasmid DNA) was pulled out using a needle. Ethidium bromide was then removed from the preparation by washing six times with equal volumes of 1-butanol. Finally, the plasmid phase was dialyzed against TE pH 8.0 overnight and the concentration of DNA was determined by measuring A_{260} and A_{280}.

Preparation of M13 RF DNA

The alkaline-lysis method was used for extraction of M13 RF DNA,

as described above. The RF DNA was purified with polyethylene glycol (PEG) as follows: The extracted DNA was dissolved in 900 µl of TE, and 600 µl of 20% PEG, 2.5 M NaCl was added, placed on ice for 1 hour, centrifuged at 12000 rpm for 10 min, washed twice with 70% ethanol, and resuspended in 100 µl TE.

Preparation of M13 single stranded DNA

One hundred µl of an overnight culture of JM105 or XL1-Blue and a single isolated plaque, obtained from transfection, were added to LB medium, and incubated for 6 hrs at 37°C, with shaking. This was then centrifuged for 30 min at 15000 rpm, 1/5 volume of PEG solution (10% PEG 8000, 2.5 M NaCl) was added to the supernatant, mixed, set on ice for 30 minutes, centrifuged, and the pellet was dried overnight at 4°C. It was then resuspended in phenol extraction buffer (100 mM Tris-HCl, 300 mM NaCl, 1 mM EDTA). The phage were lysed using SDS and proteinase K, extracted twice with phenol and once with chloroform. The single stranded DNA was precipitated by the addition of two volumes of 95% ethanol, placing at -20°C for 1 hr, centrifugation, and washing with 70% ethanol. Finally, it was resuspended in 50 µl dH_2O.

Isolation of Yeast Genomic DNA

For this purpose the method of Rose et al. (1990) was used. The cells were grown overnight, centrifuged, and resuspended in 0.9 M sorbitol, 0.1 M EDTA (pH 7.5). Then 0.25 mg of Zymolyase 100000 was added and incubated at 37°C for 60 minutes. The cells, now spheroblasts, were centrifuged and resuspended in 50 mM Tris-Cl,

20 mM EDTA, and SDS was added to a concentration of 1%, and incubated for 30 min. at 65°C. Potassium acetate was added and the preparation was stored on ice for 1 hr, centrifuged, and the supernatant transferred to another tube, in which the DNA was ethanol precipitated. The pellet was resuspended in TE, RNase treated, repelleted, dried, and redissolved in TE for storage until needed.

In vitro Site-Directed Mutagenesis

The methods of Kunkel (1985) and Stewart et al. (1985) were used for this purpose.

A 200 µl aliquot of an overnight culture of BW313 (*dut ung*, bacterial strain lacking UTPase activity) was used to inoculate 10 ml of LB and this was incubated at 37°C until log phase (as described in Kunkel 1985). Then 200 µl of this culture was added to 25 ml LB along with a single plaque of the M13, which contained the $tRNA^{Trp}$ gene putatively mutagenized, and this was incubated at 37°C for 8 hrs., with shaking. The cells were pelleted and the single stranded DNA of M13 was prepared as described earlier in this chapter.

The mutagenesis protocol utilized the double priming method of Stewart et al. (1985). First, both the mutation-containing primer (4 pmoles) and the upstream primer (2 pmoles) were kinased using 1 µl of 10mM ATP, 0.9 µl of the 10X kinase buffer (100 mM $MgCl_2$, 100 mM Tris-Cl pH 7.5, and 1 mg/ml gelatin), 0.9 µl of ß-mercaptoethanol (60 mM), and 1 µl of T4 polynucleotide kinase, and incubating at 37°C for 45 min. Then 1 µl of the single stranded DNA described in part A and 1 µl of 0.5 M NaCl were added to the kinased primers,

boiled for 2 min and cooled slowly to room temperature in a Tempblok to let the annealing reaction happen completely. Then the primers were extended using 1 unit of Klenow and ligated with 0.2 unit of T4 ligase in a tube containing 20 mM Tris-Cl, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, and 1 mM of each dNTPs. The reaction was incubated at RT°C for 20 min and then at 15°C overnight. Next day, a transfection was done using half of the above reaction. Plaque hybridization was used in order to find the plaque having the mutated sequence.

Plaque and Colony Hybridization

For plaque or colony hybridization, the transfection or transformation plates were cooled at 4°C for 30 min. Then a piece of nitrocellulose was placed on the plate, lifted after 2 min, and placed on a 3 MM paper soaked in denaturing solution, 0.5 M NaOH/1.5 M NaCl, for 5 min. The filter was then transferred to a tray containing neutralization solution, 0.5 M Tris-Cl pH 7.0/ 3 M NaCl, for 10 min. Filters were air dried on paper towels and baked in 80°C oven for 2 hrs. The filters were prehybridized in 4 X SET (0.6 M NaCl, 0.12 M Tris.Cl pH 8.0, 8 mM EDTA), 10X Denhardts (0.2% (w/v) ficol, 0.2% (w/v) polyvinyl pyroliodine, 0.2% (w/v) bovine serum albumin), 0.1% SDS, 0.1% Na_2PP_i for 30 min at 42°C, and hybridized in the same solution with appropriate end-labeled oligonucleotide for 1 hr at 42°C, 1 hr at 37°C, and then 30 min at RT. Washing was done in 4X SSC/0.1% SDS for 5 minutes and then in 4X SSC for 10 min. Finally, the filters were wrapped in Saran Wrap and exposed to X-ray film (X-OMAT KODAK) at -70°C overnight.

Preparation of 5' end-labeled Probes

In order to maximize specific activity, the end labeling was done in a molar ratio of 4:1 of [γ-^{32}P]ATP to oligonucleotide. The end-labeling reaction contained 2.5 pmol of oligonucleotide, 5 µl of [γ-^{32}P]ATP (4500 µci/µl), 5 µl 10X kinase buffer, 5 µl 60 mM ß-mercaptoethanol, and 5 units of T4 polynucleotide kinase in a final volume of 50 µl. The tube was incubated at 37°C for 45 minutes, and the unincorporated [γ-^{32}P]ATP was excluded from the probe using spun-column chromatography through Sephadex G-25 or G-50.

Southern Blotting

For the Southern blotting of the yeast genomic DNA 5 µg of DNA ,and for the PCR products 0.5-1 µg of DNA, were run on a 1.5-2 % agarose gel. The gel was denatured twice in 0.5 M NaOH/1.5 M NaCl each time for 30 minutes, and then neutralized in 0.5 M Tris-Cl/1.5 M NaCl. Nylon membrane (GeneScreen plus) was used to transfer DNA to the membrane overnight using 10X SSC (1.5 M NaCl, 0.15 M Sodium Citrate). After drying the membrane at room temperature for at least 2 hrs, it was prehybridized for 1 hr at 42°C. Probe was added to the tube to a final concentration of approximately 10^6 CPM/ml and hybridization was done overnight at 42°C. The hybridization solution was 50% formamide, 1 M NaCl, 50 mM PIPES pH 7.0, 0.5% SDS, 5X Denhardt's reagent, 10 mM EDTA pH 8.0, and10 µg/ml Salmon sperm DNA. The next day the membrane was washed twice in 2X SSC at RT for 30 min., twice in 2X SSC/1% SDS at 55°C for 30 min, and twice in 0.1x SSC at RT for 15 minutes. Finally the

membrane was exposed to X-ray film for 1-2 days at -70°C.

Polymerase Chain Reaction (PCR)

In order to amplify the flanking sequences corresponding to the yeast $tRNA^{Trp}$ genes (Figure 7), 1 µg of the yeast genomic DNA was digested with different restriction enzymes; *Sau*3A, *Hpa*II, *Rsa*I, *Hin*fI, *Hae*III, *Hin*PI. Then 10^{-1} and 10^{-2} dilutions were made for each reaction, and the fragments were religated by ligating overnight either at room temperature or 15°C. The following day the oligonucleotide primers complementary to the 5' and 3' ends of the $tRNA^{Trp}$ gene were added to the ligation reactions to a final concentration of approximately 250 nM. The reaction was carried out in a total volume of 100 µl, containing 10 µl of 10X buffer (700 mM Tris-Cl pH 8.8, 20 mM $MgCl_2$, 1% Triton X-100), dNTPs each to a final concentration of 0.2 mM, and 1 unit of Taq DNA polymerase. The template DNA was initialy denatured at 94°C for 4 min. In the following amplification cycles the denaturing condition was 30 seconds at 94°C. Then the primers and templates were annealed at either 55°C or 65°C for 30 seconds in each cycle, and the primers were extended at 75°C for 3 minutes in each cycle for a total of 25 cycles. The amplified products were electrophoresed on a 1-2% agarose gel, then agarose plugs containing the product were cut out of the gel, treated with Geneclean (as described by the manufacturer), and reamplified when necessary. However, the annealing temperature was lowered to 55°C and the PCR reactions were done for 15 cycles.

Sequencing

1-ssDNA sequencing: Where the tRNA genes or PCR fragments were subcloned in M13mp18 or 19, the ssDNA was extracted as described, and sequenced using Sequenase Version 2.0 (obtained from United States Bichemical Corporation). The template ssDNA was annealed to the universal primer by heating to 65°C for 2 min and then allowed to cool down slowly to 30°C in a Tempblok. The primers were extended and labeled with diluted Sequenase by adding diluted labeling mix, and 0.5 µl of [α-^{35}S]dATP, and then stopped by adding ddNTPs. The reactions were electrophoresed on a 6% polyacrylamide/urea denaturing gel. The gels were dried under heat and vacuum and then exposed to X-OMAT kodak film overnight at RT.

2-PCR products: The fragments which had been amplified by PCR were sequenced either by cloning into M13mp19 and then sequencing as described above, or directly after being amplified by PCR. One to two µg of each PCR product was electrophoresed on an agarose gel until the band was distinctly separated from the primers, and excised and prepared as described. A 100 ng aliquot of the sequencing primer was added and the template-primer mix was boiled for 5 min, and then frozen immediately on dry ice in order to get an appropriate amount of primer-template annealing. One µl of 0.1 M DTT, 1 µl of the labeling mix, 0.5 µl of Mn^{++} (0.15 M Sodium Isocitrate, 0.1 M $MnCl_2$)buffer, 0.5 µl of [α-^{35}S] dATP, and 3 units of Sequenase Version 2.0 were added to the mix and incubated at RT

for 2 min. The extension was stopped by adding the mix to termination tubes containing ddNTPs and spinning down at the same time. The reactions were then electrophoresed on a polyacrylamide gel and exposed to film.

Tetrad Analysis

For analysis of yeast tetrads following sporulation, cells of opposite mating types were crossed, mixed on a YEPD plate, and incubated at 30°C for 5 hrs (Rose et al. 1990). The diploid cells were picked up using a glass needle and micromanipulator, while working under a dissecting microscope and plated on YEPD for two days. The diploid cells were plated on presporulation media for 2 days at RT, and then on sporulation media (Rose et al. 1990) and incubated at RT until they began to sporulate, usually 5-10 days. In order to prepare tetrads for dissection, a toothpick-swab of the sporulating diploid cells was washed in 300 µl of sterile water, 3 µl of diluted glusulase (1/10 dilution, obtained from Sigma) was added, mixed, and incubated at RT for 10 min. The tetrads were immediately dissected as described (Rose et al. 1990). The separated spores were plated on YEPD at 30°C for two days and were replica plated onto a medium lacking tryptophan.

In vivo Suppression Assay

The suppressor ability of the tRNA constructs was tested either by streaking them onto the indicator media or by a drop test (Shaw and Olson 1984). For the drop test, single colonies of the transformed yeasts were picked from ura$^-$ plate, added to 5 ml liquid medium

lacking uracil (Rose et al. 1990), and incubated for up to 48 hrs at 30°C with shaking until the desired concentration (10^7-10^8 cells/ml) was reached as measured by OD_{600}. Then different dilutions to 10^{-4} of the culture were made and 20 µl of the dilutions were dropped on the suppressor indicator plates, and the plates were incubated at 30°C.

Growth Kinetics in Hypertonic Media

The growth kinetics of some of the transformed yeast strains, were assayed by incubating the cells in ura$^-$ media overnight (10^7 cells/ml) and then adding it to ura$^-$ lys$^-$ liquid media containing 0 M, 1 M, or 2 M KCl, to a final concentration of 10^5 cells/ml and incubating at 30°C with shaking. An aliquot of media was used to measure OD_{600} each 5 hrs. The concentration of cells was also determined by plating an aliquot of the media on a YEPD plate.

RESULTS

As mentioned in the introduction, two goals were pursued in the present work. First, attempts were made to construct an ochre suppressor $tRNA^{Trp}$ gene without an intron, and then the behavior of the constructed gene as an ochre suppressor was studied *in vivo*. Secondly, the 5' and 3' flanking regions of the $tRNA^{Trp}$ genes were amplified and a number of them were sequenced.

1- oc1ΔIVS$tRNA^{Trp}$ Gene Construction and Characterization

Cloning and Sequencing of the $tRNA^{Trp}$ Gene

A 0.41 Kb *Hin*cII-*Hae*III fragment of yeast DNA from plasmid 2BTrp (Kang et al. 1979), containing a yeast $tRNA^{Trp}$ gene, was previously subcloned into M13mp19 using *Eco*R1 linkers. In addition, a CCA to TTA change in the anticodon of this gene had already been constructed (Atkin 1992). For further oligonucleotide-directed *in vitro* mutagenesis with the available deletion-containing oligonucleotide, the M13mp19 containing the altered $tRNA^{Trp}$ (ochre suppressor $tRNA^{Trp}$) gene was digested with *Eco*R1 and religated. ssDNA was prepared and the insert was sequenced to make sure that no other mutations had occurred in the gene.

Deletion of Intron by *in vitro* Mutagenesis

In order to remove the intron of the putative ochre suppressor $tRNA^{Trp}$ gene, the oligonucleotide-directed mutagenesis methods of Kunkel (1985) and Stewart et al. (1985) were used. First, ssDNA was

prepared from M13mp19, with the $tRNA^{Trp}$oc1 gene subcloned into its *Eco*R1 site, by growing it in an *E. coli* dut⁻ ung⁻ strain, BW313 (Kunkel 1985). A 20 bp oligonucleotide (AAT5) was designed to be complementary to the mature ochre $tRNA^{Trp}$ and bind to 13 bp 5' and 7 bp 3' to the position of the intron (figure 1). A second oligonucleotide was used which anneals upstream (-28 to -13) of the start site of the tRNA coding region. This double primer method is reported to prevent displacement of mutant oligonucleotides from the template and increase the efficiency of the recovery of mutants (Stewart et al. 1985). Both primers were phosphorylated using T4 polynucleotide kinase, and then annealed to ssDNA template. The primers were extended using DNA polymerase I, and ligated. JM105 was then transformed with the double stranded M13 DNA. M13 plaques having the deleted tRNA gene were selected by hybridization using the oligonucleotide utilized for constructing the deletion (figure 1). About 40% of the plaques hybridized to the probe. As shown in the figure 1, a new *Dra*1 recognition site (AAATTT) resulted from deletion of the intron of the ochre suppressor $tRNA^{Trp}$ gene. In order to assure that the intervening sequences of the gene were precisely deleted while the rest of the gene was intact, single stranded M13 DNA was extracted from the plaques and then sequenced. The relevant sequences of the intron-containing and the intron-deleted $tRNA^{Trp}$ genes are shown in figure 2.

Subcloning into Yeast-*E. coli* shuttle vectors

To study the expression pattern of this mutated gene, the 376 bp

Figure 1: Oligonucleotide directed site-specific deletion of the intron. The intron of the putative ochre suppressor $tRNA^{Trp}$ gene was deleted using oligonucleotide directed mutagenesis, utilizing an oligonucleotide (AAT5) complementary to sequences flanking the intervening sequences of this gene. Deletion of the intron results in a new *Dra*1 recognition site (illustrated). The anticodon is indicated by bold letters on the antisense strand.

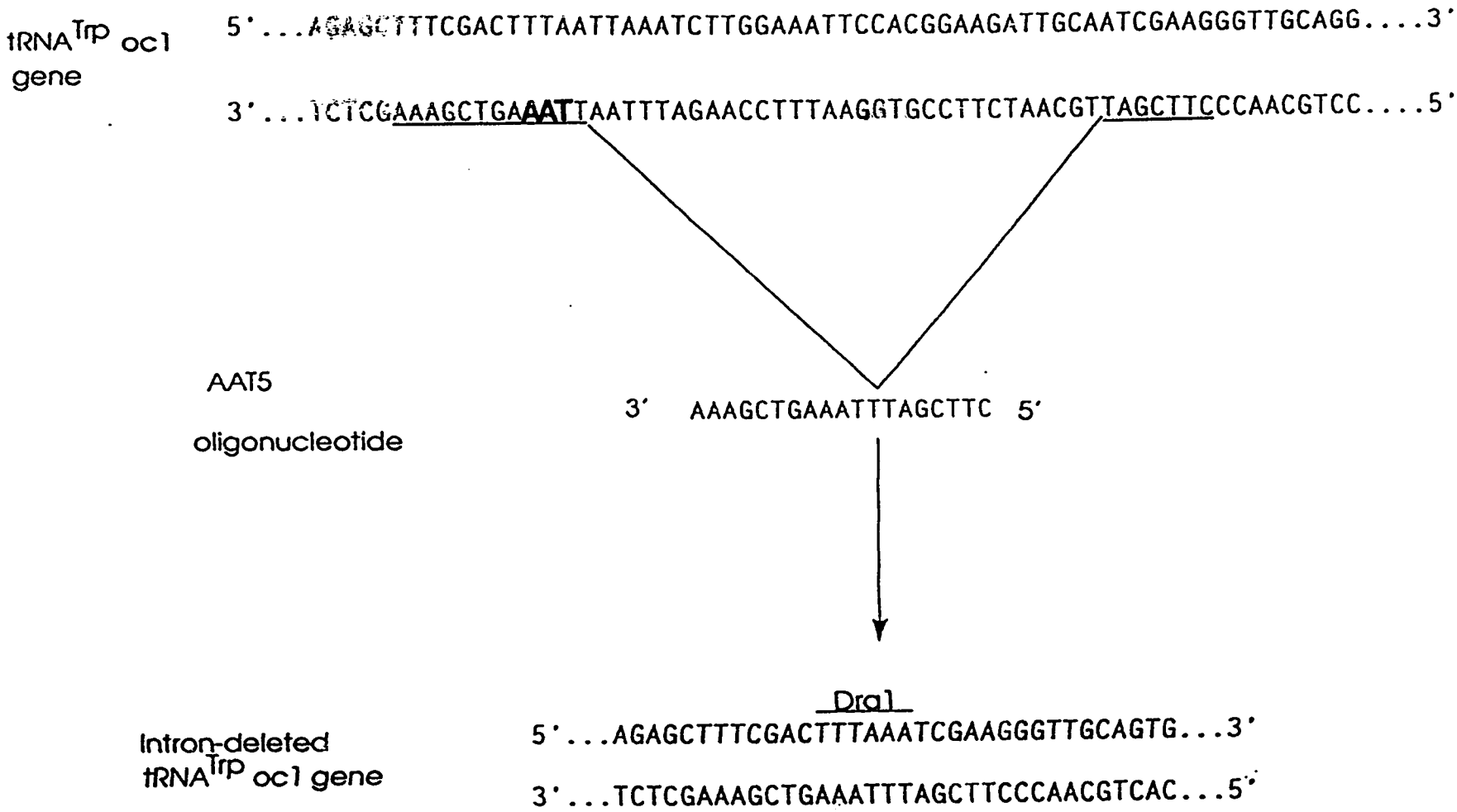
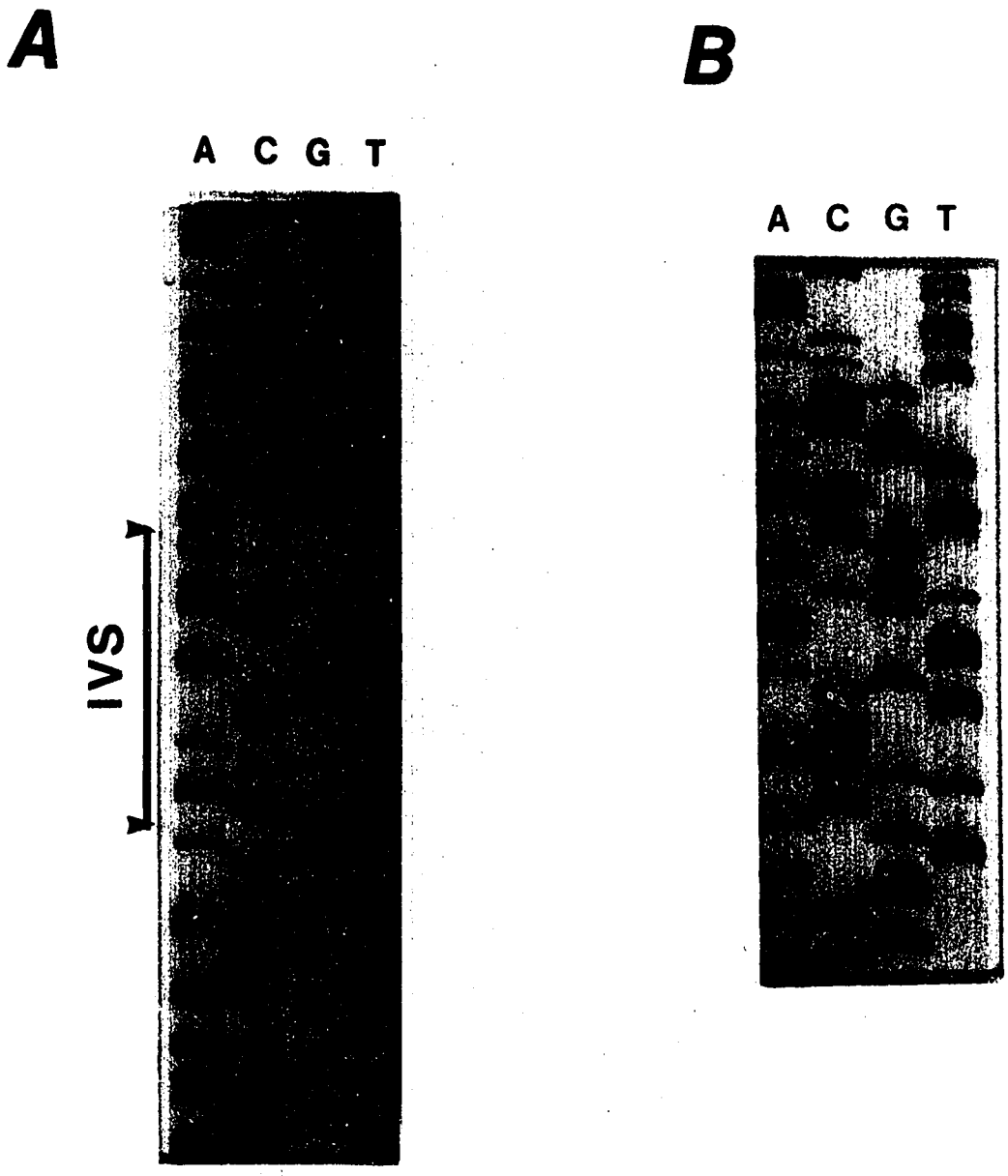

tRNA^Trp oc1 gene
5'...AGAGCTTTCGACTTTAATTAAATCTTGGAAATTCCACGGAAGATTGCAATCGAAGGGTTGCAGG....3'
3'...TCTCGAAAGCTGAAATTAATTTAGAACCTTTAAGGTGCCTTCTAACGTTAGCTTCCCAACGTCC....5'
AAT5
oligonucleotide
3' AAAGCTGAAATTTAGCTTC 5'
DraI
Intron-deleted tRNA^Trp oc1 gene
5'...AGAGCTTTCGACTTTAAATCGAAGGGTTGCAGTG...3'
3'...TCTCGAAAGCTGAAATTTAGCTTCCCAACGTCAC...5'

Figure 2: Sequences of a yeast putative ochre suppressor $tRNA^{Trp}$ gene with the requisite anticodon sequence (A), and the engineered intron-deleted form of this gene (B). The 34 bp intervening sequence common to all $tRNA^{Trp}$ genes is indicated on the figure.


A
A C G T
IVS
B
A C G T

intronless $tRNA^{Trp}$oc1 gene-containing fragment was subcloned into the *Eco*RI site of different shuttle vectors; YCp50, YRp17, and YEp352. The *E. coli* transformants which contain the recombinant YRp17 and YCp50 constructs were selected by hybridization with the AAT5 oligonucleotide. YCp50 and YRp17 are pBR322-derived plasmids containing a yeast chromosomal site capable of autonomous replication (ARS1). They have an amp^R gene for selection in *E. coli* and ura^+ and trp^+ genes as selectable markers in yeast. YCp50 also has a yeast chromosomal centromeric region so it is present in single copy in yeast (Struhl et al. 1979), whereas YRp17 is present in multiple copies. YEp352 is a pUC-derived vector and allows the identification of recombinant plasmids by screening for α-complementation of the lacZΔM15 mutation in *E. coli*. In addition, it has a 2μm circle origin of replication and is present in high copy numbers in yeast (Hill et al. 1986). The reason for choosing YEp352 was the higher stability of this plasmid in yeast compared to YRp17.

Choice of *S. cerevisiae* strains

In order to investigate the suppression ability of the intron-deleted construct, different yeast strains were chosen for transformation for an *in vivo* suppression assay (see Materials and Methods). The reason for choosing a variety of yeast strains was testing for different ochre mutations in different genetic backgrounds. It has been previously demonstrated that an intron-containing ochre suppressor $tRNA^{Trp}$ gene does not function as an ochre suppressor when introduced into yeast strain JG369-3B (Atkin 1992) even when it is present at high copy numbers in the cell. This

strain contains ochre mutations in two genes involved in adenine and lysine biosynthesis (*ade2-1* and *lys2-1*). Thus, this strain was used as a transformation host for the intron-deleted construct to investigate the functional ability new construct as an *in vivo* suppressor. However, other studies show that not all ochre mutations are suppressed with the same efficiency in the presence of a suppressor tRNA (reviewed in Hawthorne and Leupold 1974). These studies indicate that the *ade2-1* and *can1-100* alleles are difficult to suppress whereas *his5-2*, *lys1-1*, and *trp5-48* are "easier to suppress" ochre mutations (Hawthorne and Leupold 1974, Ono et al. 1979). Therefore, a second yeast strain (RVB-4B) containing *trp5-48*, *lys1-1*, and *ade2-1* was used in this experiment. The ochre mutation *trp5-48* is known to be a sensitive mutation for nonsense suppression. It can be suppressed even in the presence of ψ^+ factor when no ochre suppressor is present (Ono et al. 1986 , Cox et al. 1988).

The ψ^+ factor is known to increase the efficiency of suppression of ochre nonsense mutations by certain ochre tRNA suppressors. To investigate the effects of a ψ^+ background on nonsense suppression by the ochre suppressor constructs described herein, two ψ^+ strains (RVB-45C and AB1380) were also transformed with both the intron-containing and the intron-deleted constructs present on single and high copy number plasmids. The two yeast strains, RVB-45C and AB1380, were chosen because they harboured *lys1-1* and *lys2-1* mutations, respectively. Thus, the effect of ψ^+ could be investigated by comparing the efficiency of suppression in a ψ^+ background to that in ψ^- strains harbouring *lys1-1* and *lys2-1*, RVB-4B, and JG369-

3B, respectively.

Tetrad Analysis for the putative ψ^+ Strain

The presence of the ψ^+ factor in strain RVB-45C was tested by crossing it to a *trp5-48*-harbouring yeast strain of the opposite mating type. This mutation can be suppressed in the presence of ψ^+ factor in the absence of a tRNA suppressor. The ψ^+ tester strain (RVB-45C) itself contains a *trp1-1* mutation. Therefore, if the extrachromosomal factor ψ^+ is present in this strain, we expect to see a $2^+:2^-$ segregation on trp^- media. Different strains of yeast were used for the cross (table 1 in Materials and Methods). However, all of them sporulated poorly in my hands. Ten tetrads were finally dissected from the cross, RVB-45C [ψ^+, *trp1-1*] x XV185-6A [*trp5-48*]. All of the tetrads showed the expected $2^+:2^-$ ratio on trp^- media indicating the presence of the ψ^+ factor in this strain. The two meiotic products that failed to grow in these tetrads were presumably due to the segregation of the unsuppressed *trp1-1* allele, whereas the two products that did grow were $trp1^+$ segregants in either a *trp5-48* or $trp5^+$ background.

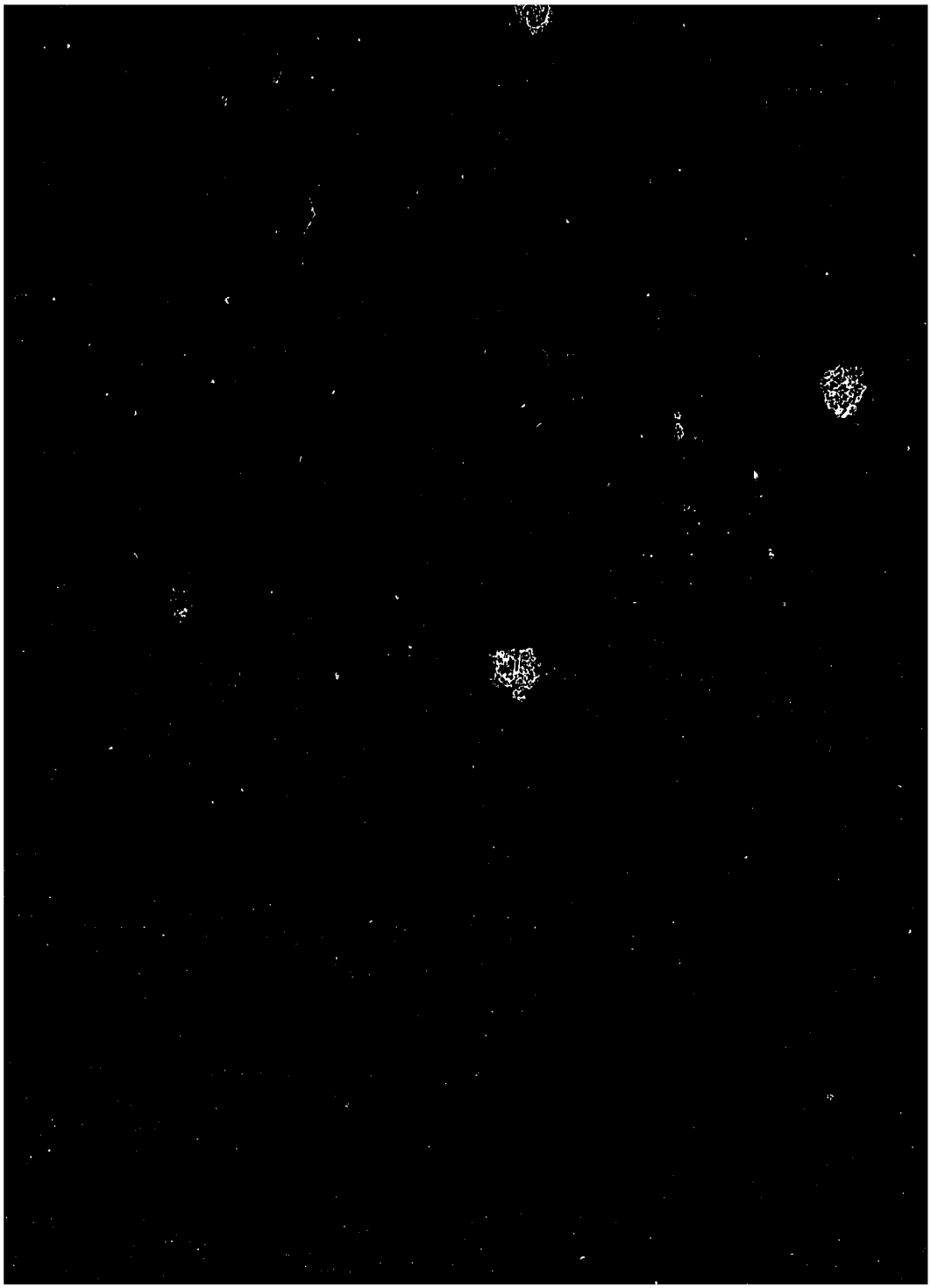
Suppression Assay

In my initial experiments, two yeast strains, JG369-3B and RVB-45C [ψ^+], both containing *ade2-1* ochre nonsense mutation were transformed with YRp17, YCp50, pYR17oc1 (ochre suppressor $tRNA^{Trp}$ cloned into YRp17), pYRoc1ΔIVS (intron-deleted ochre $tRNA^{Trp}$ cloned into YRp17), pYCoc1 (ochre tRNATrp cloned into YCp50), and pYCoc1ΔIVS (intron-deleted ochre $tRNA^{Trp}$ cloned into

YCp50). Transformants were selected on ura$^-$ media and, after incubation for two days at 30°C, were replica plated onto YEPD media for a color assay and onto ade$^-$, or ura$^-$ ade$^-$ omission media for a growth assay. The color of the transformants carrying an *ade2-1* mutation is an indication of the action of the suppressor. Nonsuppressed *ade2-1* yeasts are red because of the accumulation of an intermediate in adenine biosynthesis, while they turn white as the result of suppression. All of the transformants in this experiment turned red after being replica plated onto a YEPD plate, except those using the pYRoc1ΔIVS construct put into the strain having a ψ^+ factor (RVB-45C). These cells remained white for a few days but finally turned red. When the red yeast transformants were replica plated back to a ura$^-$ media only those transformants transformed with the constructs cloned into YCp50 were still able to grow slightly. This result indicates plasmid loss, and loss was especially severe when cells were transformed with the genes cloned into YRp17. In addition, no growth was seen on the ade$^-$, or ura$^-$ ade$^-$ omission media. However, a color gradient from red to almost white was seen for the RVB-45C [ψ^+] transformants (figure 3) on ura$^-$ media, with pYCoc1 transformants forming red colonies and the pYRoc1ΔIVS transformants being almost white. All of this suggested that suppression was absent or very weak, complicated by plasmid loss.

It was then decided to use a high copy number plasmid with a higher stability. Therefore, the tRNA^{Trp} genes were cloned into a 2μm circle-based plasmid, YEp352, which is present in high copy number and also which exhibits high mitotic stability (Hill et al. 1986). The suppression ability of the transformants was retested

Figure 3: Suppressor phenotype of the yeast strain, RVB-45C [ψ^+], transformed with the constructed genes.Suppressor phenotype of the yeast strain, RVB-45C [ψ^+], transformed with 1- pYCoc1 (single copy plasmid containing oc1tRNA^{Trp} gene), 2- pYCoc1ΔIVS (single copy plasmid harbouring intronless oc1tRNA^{Trp} gene), 3- pYRoc1 (multiple copy plasmid harbouring oc1tRNA^{Trp} gene), 4- pYRoc1ΔIVS (multiple copy plasmid harbouring intronless oc1tRNA^{Trp} gene) on ura$^-$ media. The cells were also transformed with YRp17 and YCp50 plasmids and all of the transformants were red (not shown in this figure).



with a drop test as well as simply streaking them on different omission media. Two ψ^- strains (JG369-3B, and RVB-4B) and two ψ^+ strains (RVB-45C, and AB1380) were used in these experiments. The results are presented in table 2. For JG369-3B, suppression was observed only when it was transformed with pYEoc1ΔIVS (figure 4A). It was also found out that the *lys2-1* ochre mutation present in this strain is suppressed easier than *ade2-1* (table 2). This is consistent with what has been found by others (reviewed in Hawthorne and Leupold 1974). Not much difference was seen in the suppression of *lys2-1* and *ade2-1* in the presence of the ψ^+ factor in AB1380. The ochre mutation, *trp5-48*, in the RVB-4B strain was also suppressed only when transformed with the intron-deleted $tRNA^{Trp}$ gene present in both single copy and high copy number plasmids (figure 4C).

The best suppression was seen for the *lys1-1* ochre mutation in RVB-45C [ψ^+] when it was transformed with the intron-deleted $tRNA^{Trp}$ gene present in high copy number. In contrast, the *lys1-1* mutation in a ψ^- background was hardly suppressed at all. When the RVB-4B [*lys1-1*] transformants were replica plated onto a ura^- lys^- media only the ones transformed with pYEoc1ΔIVS exhibited slight growth after 15-20 days, whereas RVB-45C [*lys1-1*,ψ^+] transformants transformed with the same construct exhibited strong growth on ura^- lys^- media after only 3-5 days. The effect of the ψ^+ factor on growth via enhanced suppression was quantitated using a drop test. An approximate two orders of magnitude difference in growth on lysine omission media was seen between RVB-4B transformed with pYEoc1ΔIVS and RVB-45C [ψ^+] transformed with the same construct

Figure 4: *In vivo* suppression assay of the ochre suppressor $tRNA^{Trp}$ constructs.

Plate A shows the growth of untransformed JG369-3B [*lys1-1*] cells on a ura^- lys^- plate (1) as well as the growth of JG369-3B cells transformed with pYE w.t. (2), pYEoc1 (3), pYEoc1ΔIVS (4), pYCoc1 (5), and pYCoc1ΔIVS (6) on this medium.

Plates B and C show the growth of RVB-4B [*trp5-48*] transformants in concentrations ranging from 10 to 10^5 cells on complete medium (B), and ura^- trp^- medium (C). Thus, plate A assays for the suppression of *lys1-1* (UAA), and plate C assays for the suppression of *trp5-48* (UAA).

The numbers on plates B and C denote the same constructs as plate A.

B
C

(figure 5). To investigate if the difference in growth was due to a different genetic background or only because of the action of the ψ^+ factor present in RVB-45C transformants, growth kinetics of RVB-45C transformants in hypertonic media was studied.

The codon specificity of the constructed suppressor was studied both by replica plating and by drop tests on ura$^-$ trp$^-$ and ura$^-$ met$^-$ plates. No suppression of the amber nonsense mutations (*trp1-1*, and *met8-1*) present in JG369-3B was observed, suggesting that the suppressor acts in an codon specific manner. The codon specifity of ochre suppressors in *S. cerevisiae* has been suggested to be due to the modification of 5' U in the anticodon of these tRNAs (Laten 1983), thus not allowing a wobble base pairing between this base and the 3' G present in amber nonsense codons. The codon specificity described above for the intron-deleted $tRNA^{Trp}$ gene appears to be consistent with models on codon specificity. This will be further discussed in the next section.

Growth Kinetics in Hypertonic Media

Singh et al. (1979) showed that the biological activity of the ψ^+ factor is eliminated in hypertonic media containing KCl or ethylene glycol. This observation has been exploited in the present work to demonstrate the effect of the ψ^+ factor on the suppressor function of the *in vitro*-constructed intron-deleted ochre suppressor $tRNA^{Trp}$ gene. As previously mentioned, the drop test indicated that the presence or absence of the ψ^+ factor affected the efficiency of ochre suppression by about two orders of magnitude. To prove that the

Table 2: suppressor phenotype of the yeast transformants*

Tranformants	**Mutation	*lys1-1*	*lys2-1*	*trp5-48*	*ade2-1*	*trp1-1*	*met8-1*
JG369-3B[ψ⁻]:	YEp352		-		-	-	-
	pYEoc1		-		-	-	-
	pYCoc1		-		-	-	-
	pYEoc1ΔIVS		++		+	-	-
	pYCoc1ΔIVS		-		-	-	-
RVB-4B[ψ⁻]:	YEp352	-		-	-		
	pYEoc1	-		-	-		
	pYCoc1	-		-	-		
	pYEoc1ΔIVS	+		++	+		
	pYCoc1ΔIVS	-		+	-		
RVB-45C[ψ⁺]:	YEp352	-			-	-	
	pYEoc1	-			-	-	
	pYCoc1	-			-	-	
	pYEoc1ΔIVS	+++			+	-	
	pYCoc1ΔIVS	-			-	-	
AB1380[ψ⁺]:	YEp352		-		-	-	

* Suppressor phenotype of the *S. cerevisiae* strains (JG369-3B, RVB-4B, RVB-45C, and AB1380) when transformed with the (YCp50) or multiple copy (YRp17, and YEp352) plasmids containing wild type $tRNA^{Trp}$, $oc1tRNA^{Trp}$, and $oc1\Delta IVStRNA^{Trp}$ genes.

Suppression efficiency is indicated by (+) or (-) symbols based on the growth on the ade^-, lys^-, trp^-, or met^- omission media.

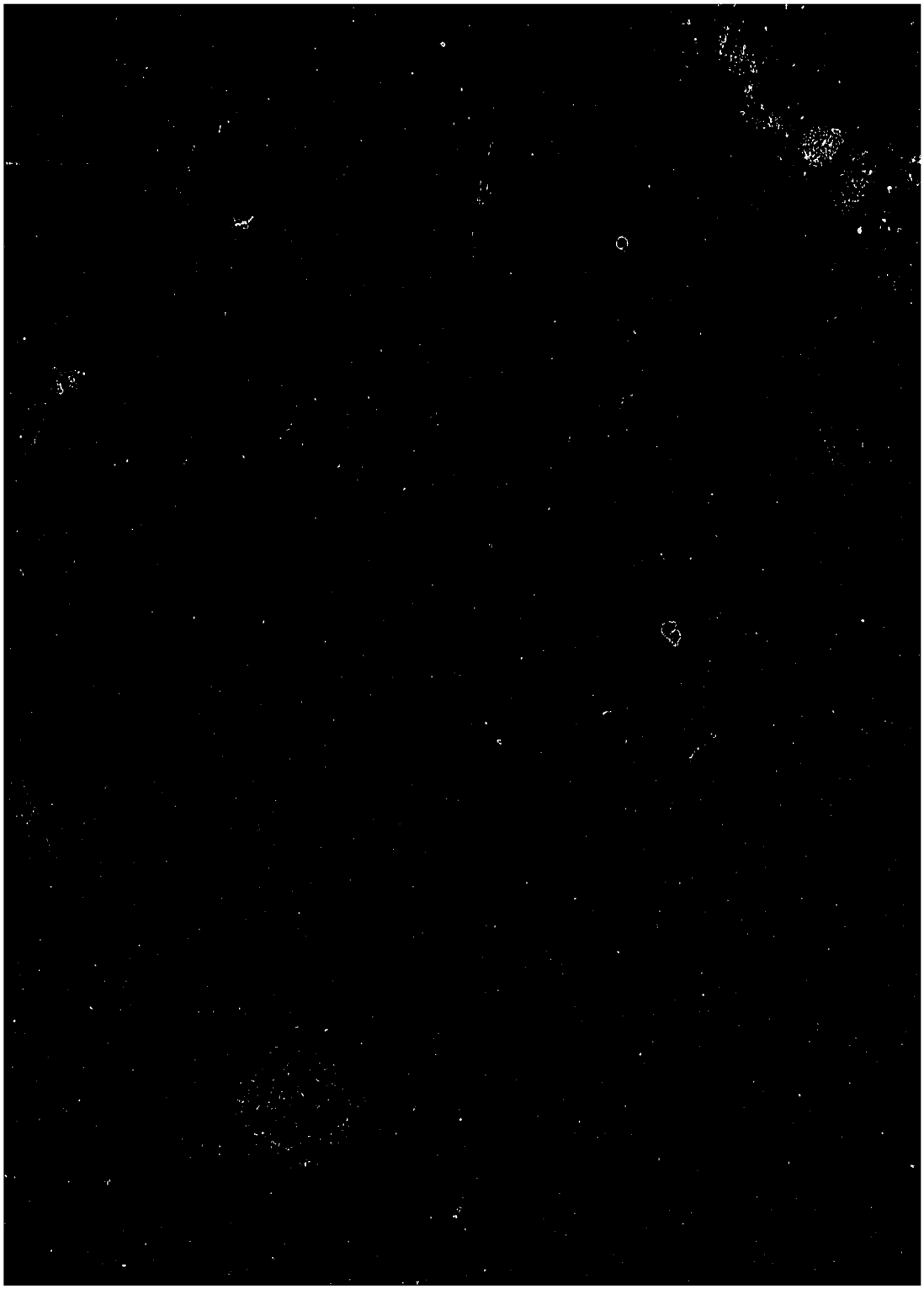
The (+++) symbol indicates a relatively strong suppressor phenotype. The cells of this group grew after 3-5 days at 30°C. The (++) symbol indicates a moderate suppressor phenotype. These cells grew after 7-10 days at 30°C.

The (+) symbol stands for low suppressor phenotype and growth after 15-20 days at 30°C. The (-) symbol indicates that no suppression (no growth) was observed, even after an extended period of incubation.

** *lys1-1*, *lys2-1*, *trp5-48*, and *ade2-1* are ochre (UAA) mutations while *met8-1*, and *trp1-1* are amber (UAG) mutations.

Shaded areas indicate the absence of the mutations in the yeast strains.

Figure 5: The enhancement of ochre suppression by the ψ^+ factor.
A: Growth of RVB-4B[ψ^-] cells, transformed with pYEoc1ΔIVS, and plated on ura$^-$ lys$^-$ medium, as the result of suppression.
B: Growth of RVB-45C [ψ^+] cells, transformed with pYEoc1ΔIVS, and plated on ura$^-$ lys$^-$ medium, as the result of suppression.

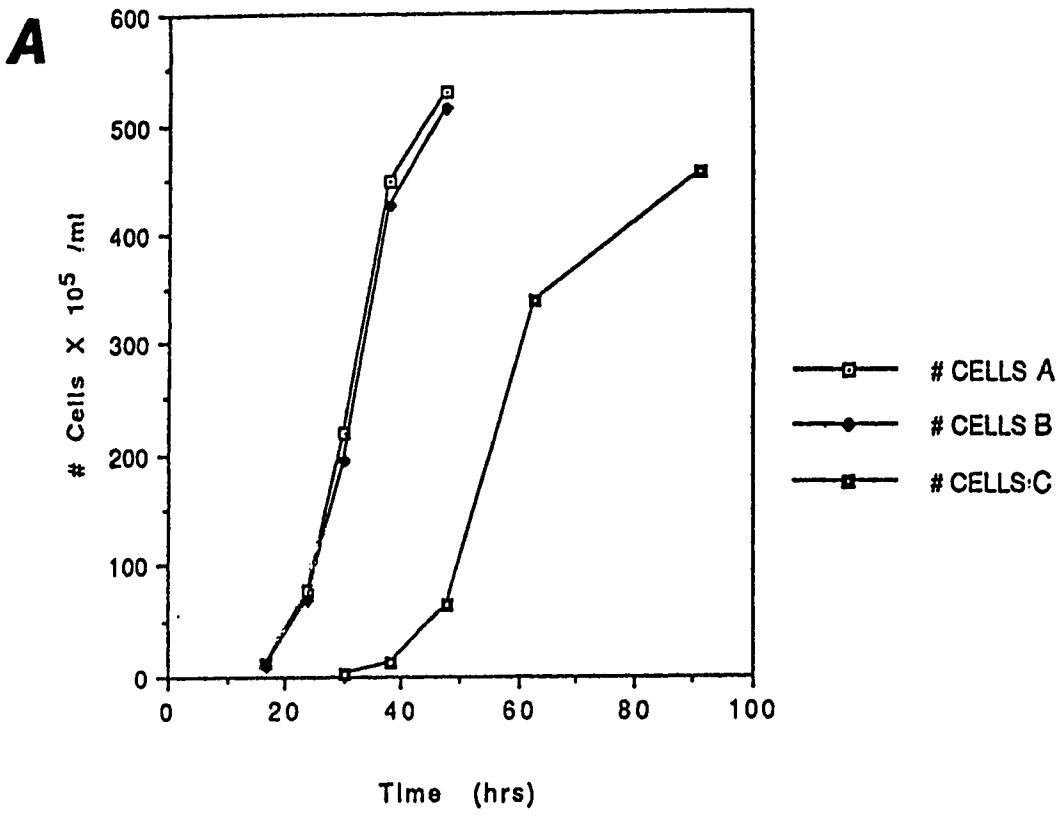


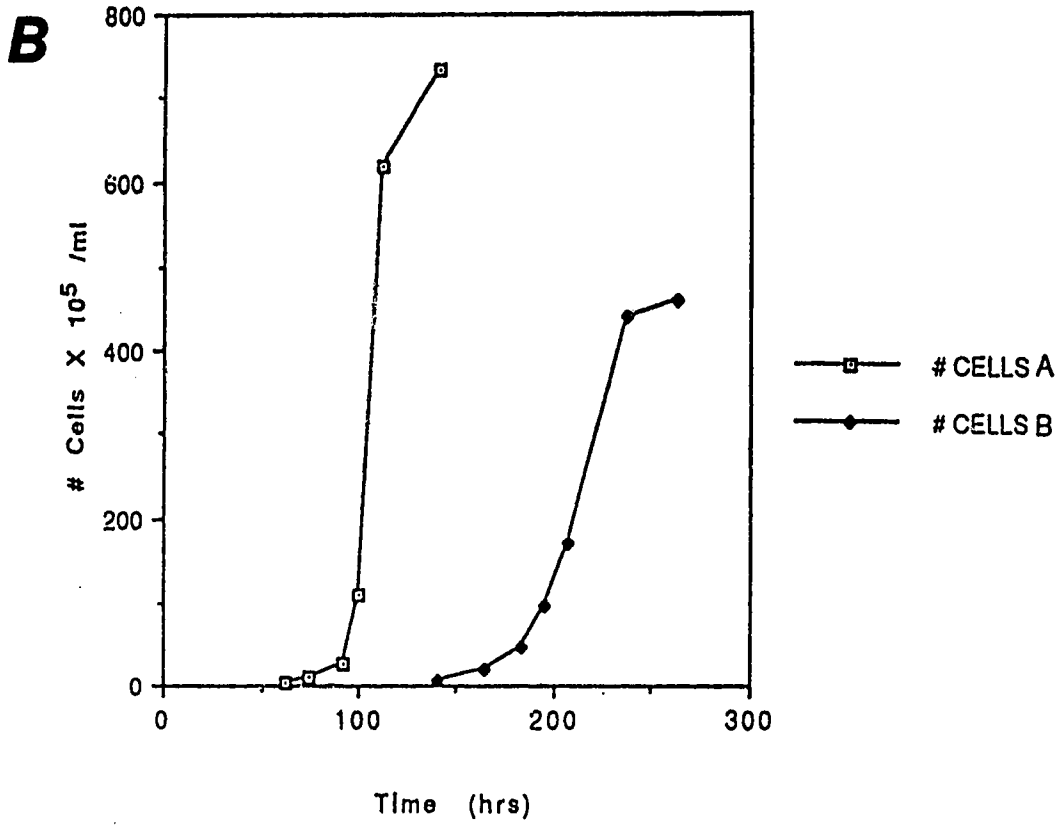
difference was not due to the different genetic backgrounds of the RVB-45C [ψ^+] and RVB-4B strains, the ψ^+ factor of RVB-45C was eliminated in hypertonic media, and the suppressor function was studied concomitantly. In order to eliminate the ψ^+ factor, RVB-45C [ψ^+] transformants transformed with pYEoc1ΔIVS were grown in ura$^-$ lys$^-$ liquid media containing increasing concentrations of KCl; 0, 1, and 2 M. To be able to grow in these media, suppression of the *lys1-1* allele must occur. The cultures were incubated at 30°C with shaking, and growth was assayed by taking samples from the culture and measuring A_{600}, as well as plating a dilution of the culture on YEPD medium. A prototrophic ura$^+$ lys$^+$ yeast strain, JG113-5R, was also grown in similar media as a control, in order to correct for the possible inhibitory effect of the hypertonic media on cell growth. The control strain started to grow in the 1 M, and 2 M KCl media after a short delay and then grew with a doubling time close to that in the 0 M KCl medium (figure 6). The ψ^+ containing strain transformed with pYEoc1ΔIVS grew well when no KCl was added. However, in 1 M KCl-containing media a long delay was seen and the doubling time was much longer in the 0 M KCl media. In 2 M KCl media no growth was seen, even after 12 days (figure 6). The experiment was stopped at this point because of the very low survival rate of the cells in 2 M KCl-containing media. The difference in doubling time of the two strains at 0 M KCl may simply reflect prototrophic growth versus growth due to weak suppression.

Study of the Nature of the ψ^+ Factor

A significant effect of the ψ^+ factor on the suppression ability of

Figure 6: Growth kinetics of a prototrophic ψ^- and an auxotrophic ψ^+ strain (transformed with the intronless ochre suppressor gene) in hypertonic ura$^-$ lys$^-$ media. Panel A shows the growth kinetics of JG369-3B [ura$^+$ lys$^+$] cells, used as a control, in ura$^-$ lys$^-$ media containing 0 M (A), 1 M (B), and 2 M (C) of KCl. Panel B illustrates the growth kinetics of RVB-45C [ψ^+, ura$^-$ lys$^-$] transformed with pYEoc1ΔIVS in ura$^-$ lys$^-$ media containing 0 M (A), and 1 M KCl (B). No growth was observed in ura$^-$ lys$^-$ medium containing 2 M KCl.


A
600
500
400
300
200
100
0
Cells X 10^5 /ml
0
20
40
60
80
100
Time (hrs)
CELLS A
CELLS B
CELLS C


B
800
600
400
200
0
Cells X 10^5 /ml
0
100
200
300
Time (hrs)
CELLS A
CELLS B

the intron-deleted $tRNA^{Trp}$ gene was demonstrated by the above-mentioned experiments. As mentioned in the introduction, the ψ^+ factor determinant and the way it enhances the efficiency of ochre suppressors is still unknown. Since temperature sensitivity can give a hint to further understanding the function of a gene (Pakula and Sauer 1989), I investigated the nature of ψ^+, by observing the heat sensitivity of RVB-45 [ψ^+] cells transformed with pYEoc1ΔIVS. This was done by plating different concentrations of the cells (10^3, 10^4, and 10^5) on ura^- lys^- media, and then incubating them at 30°C and 37°C. While a slower growth was seen at the higher temperature, the efficiency of suppression was similar at both temperatures (data not shown).

2-Characterization of the 5' and 3' Flanking Regions of $tRNA^{Trp}$ Genes

Previous studies have shown that the 5' and 3' flanking regions of tRNA genes are important in the transcription of these genes in different organisms (Shaw and Olson 1984, Sajjadi and Spiegelman 1989) despite having no conserved sequences. With respect to the $tRNA^{Trp}$ genes of *S. cerevisiae*, the flanking sequences of only one of the genes has been published (Kang et al. 1980). However, it has been found by PCR amplification and sequencing that the $tRNA^{Trp}$ gene sequence is identical among the six members of this family (Atkin et al. 1992a). This fact was used in this work in order to amplify the flanking regions of these genes.

Inverse PCR of the Yeast Genomic DNA

A summary diagram of inverse PCR amplification of the flanking regions for the $tRNA^{Trp}$ genes of *S. cerevisiae* is shown in figure 7.

Yeast genomic DNA was extracted from JG113-5R and was digested with different restriction enzymes; *Sau*3A, *Hpa*II, *Hin*PI, *Hae*III, and *Rsa*I. These enzymes are 4-base cutters and were selected because of having multiple restriction recognition sites in the yeast genome as well as having no recognition site within the $tRNA^{Trp}$ genes. The restricted fragments were ligated and subjected to inverse PCR amplification using primers complementary to the 5' and 3' ends of the $tRNA^{Trp}$ genes; ITrp-R (right side inverse PCR primer of $tRNA^{Trp}$ genes), and ITrp-L (left side inverse PCR primer of $tRNA^{Trp}$ genes). The sequence and the location of these primers is shown in figure 10. The products of the inverse PCR were then electrophoresed on a 1.5% agarose gel (figure 8A). As seen in the figure, the *Sau*3A-digested DNA resulted in 5 bands similar to the number of $tRNA^{Trp}$ genes in *S. cerevisiae* (six genes) determined by Atkin et al. (1992a). Occasionally, when a lower temperature of annealing was used (55°C), two other faint bands also appeared (figure 8B). To demonstrate that these fragments are the true flanking regions of the genes and not PCR artifacts, in a subsequent PCR amplification of the *Sau*3A-digested DNA, another primer (YTrpInt-R, yeast right side inverse PCR primer of $tRNA^{Trp}$ genes) was used instead of ITrp-R. This oligonucleotide was complementary to sequences within the genes (figure 10). Thus, part of the genes were intentionally amplified in the PCR reactions and the products of the PCR were probed with the oligonucleotide complementary to that

Figure 7: Schematic diagram of the strategy used to amplify the 5' and 3' flanking regions of *S. cerevisiae* $tRNA^{Trp}$ genes. The 5' and 3' primers (ITrp-L, and YTrpInt-R, respectively) are shown by arrows. Yeast genomic DNA was digested with different restriction enzymes, then DNA fragments were self-ligated, and then PCR amplification was done using the primers directing outward.

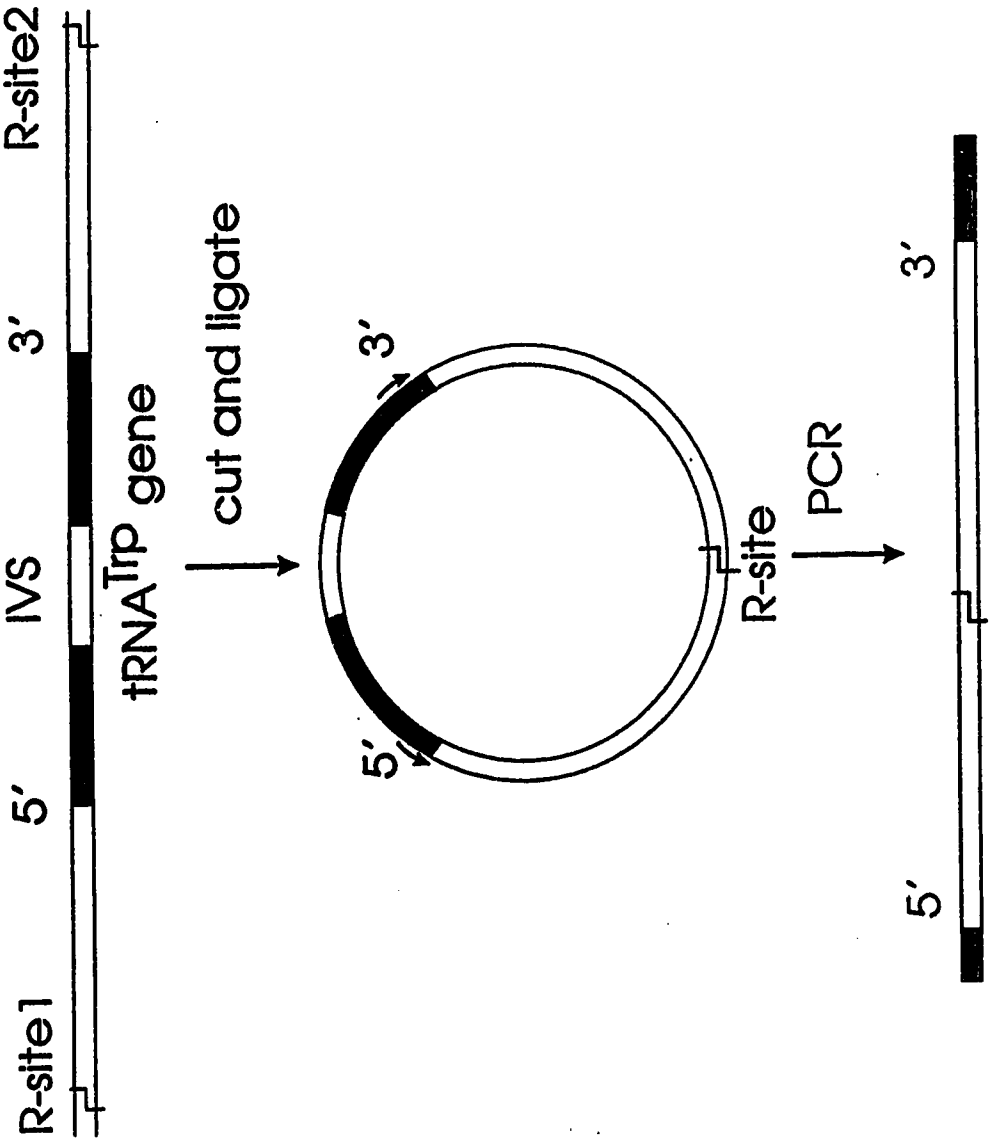

R-site1
5'
IVS
3'
R-site2
tRNA^Trp gene
cut and ligate
3'
5'
R-site
PCR
3'
5'

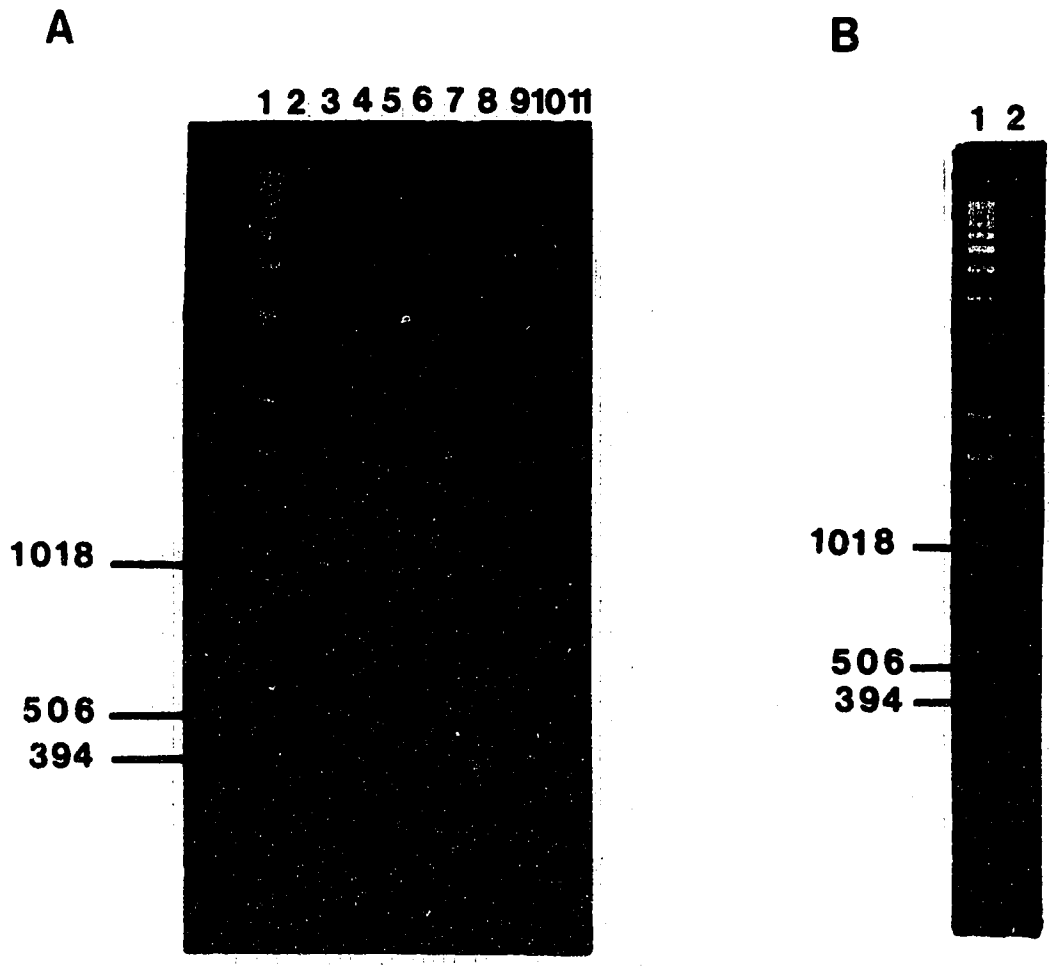
Figure 8: Inverse PCR of yeast genomic DNA.

A: Inverse PCR products of yeast genomic DNA digested with *Sau*3A (lanes 2 and 3), *Hae*III (lanes 4 and 5), *Hpa*II (lanes 6 and 7), *Rsa*I (lanes 8 and 9) and *Hin*PI (lanes 10 and 11).

For the reactions in lanes 2, 4, 6, 8, and 10, 1 μg of DNA was used while 100 ng of DNA was used for the reactions on lanes 3, 5, 7, 9, and 11. The primers used for all of these reactions were ITrp-R and ITrp-L as indicated in figure 10.

B: Inverse PCR of yeast genomic DNA digested with Sau3A (lane 2). The elongation temperature used in this experiment was 55°C.

Lane 1 in A and B indicates the size markers.


A
1 2 3 4 5 6 7 8 9 10 11
1018
506
394
B
1 2
1018
506
394

region (ITrp-R) to demonstrate that the amplified products were authentic flanking sequences. The amplification of the *Sau*3A-digested DNA using YTrpInt-R and ITrp-L resulted in five clear and distinct bands (IS1-IS5) as well as two faint bands, IS6 (1050 bp), and IS7 (1180 bp) (figure 9B). When the amplification reaction was carried out in the presence of 3% DMSO, the faint bands disappeared, although the overall amount of amplified product was also reduced.

Southern Blotting Analysis of the Amplified Fragments

The products of the inverse PCR of the *Sau*3A digestion of yeast genomic DNA were electrophoresed on a 2% agarose gel until the bands were distinctly separated. The bands were excised from the gel, purified, and then reamplified using the same primers. Despite several attempts to reamplify IS6 and IS7, no products were obtained, even though the other bands were successfully reamplified.

In order to be certain that the inverse PCR products do, in fact, represent the flanking regions of the $tRNA^{Trp}$ genes and not PCR artifacts, another Southern blotting experiment was set up. After electrophoresing the amplified products in an agarose gel and transferring the fragments to a nylon membrane (Gene Screen Plus), the membrane was hybridized with an oligonucleotide probe (ITrp-R, figure 10) complementary to the part of the $tRNA^{Trp}$ gene which was supposed to be amplified along with the flanks (figure 9B). This result indicated that the IS1-IS5 fragments were true amplifications of the flanking regions of the genes.

These results, while satisfactory, were not completely consistent with the number of $tRNA^{Trp}$ genes in *S. cerevisiae* determined by

Figure 9: Southern blot analysis of yeast genomic DNA digested with *Sau*3A, and amplified PCR products.

A-Southern analysis of yeast genomic DNA digested with *Sau*3A.

B-Southern analysis of the inverse PCR products of yeast genomic DNA digested with *Sau*3A.

Both of the Southern blots were hybridized with the ITrp-R oligonucleotide (as indicated in figure 10).

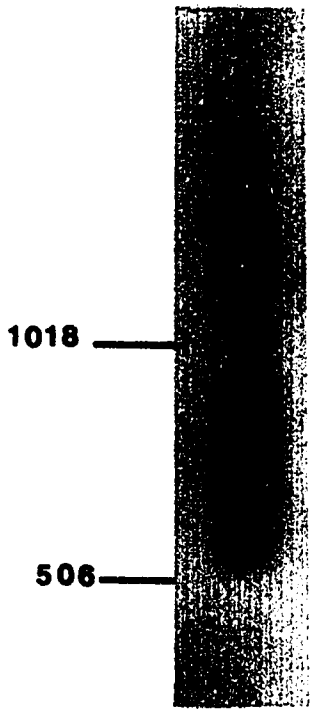
The size markers are indicated by lines.

Lane 1: The inverse PCR amplified fragments. IS1 (the smallest fragment, 500) to IS7 (the biggest fragment, 1180 bp). IS6 is approximately 1050 bp.

Lane 2: The same reaction as the lane 1, except 3% DMSO was included.

Lane 3-7 show reamplified fragments, IS1 (500 bp), IS2 (590 bp), IS3 (640 bp), IS4 (660 bp), and IS5 (770 bp), respectively.

A



B

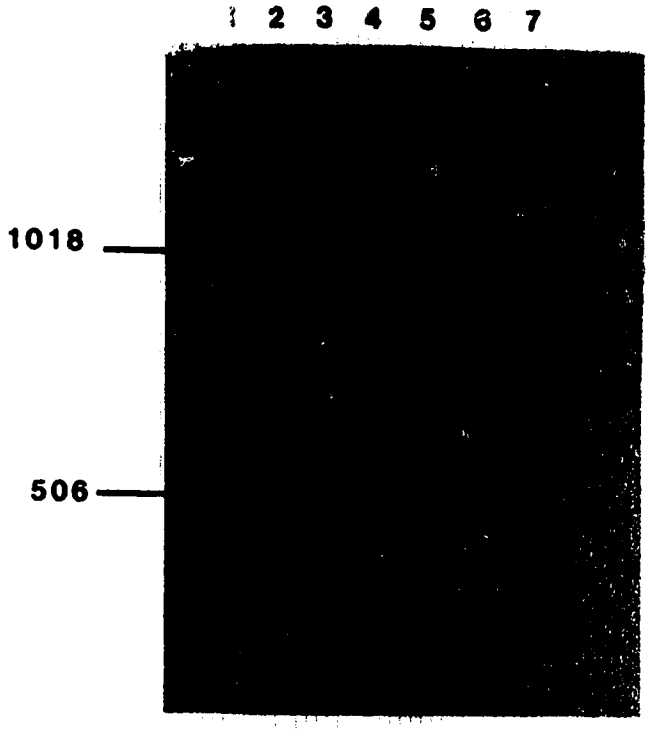
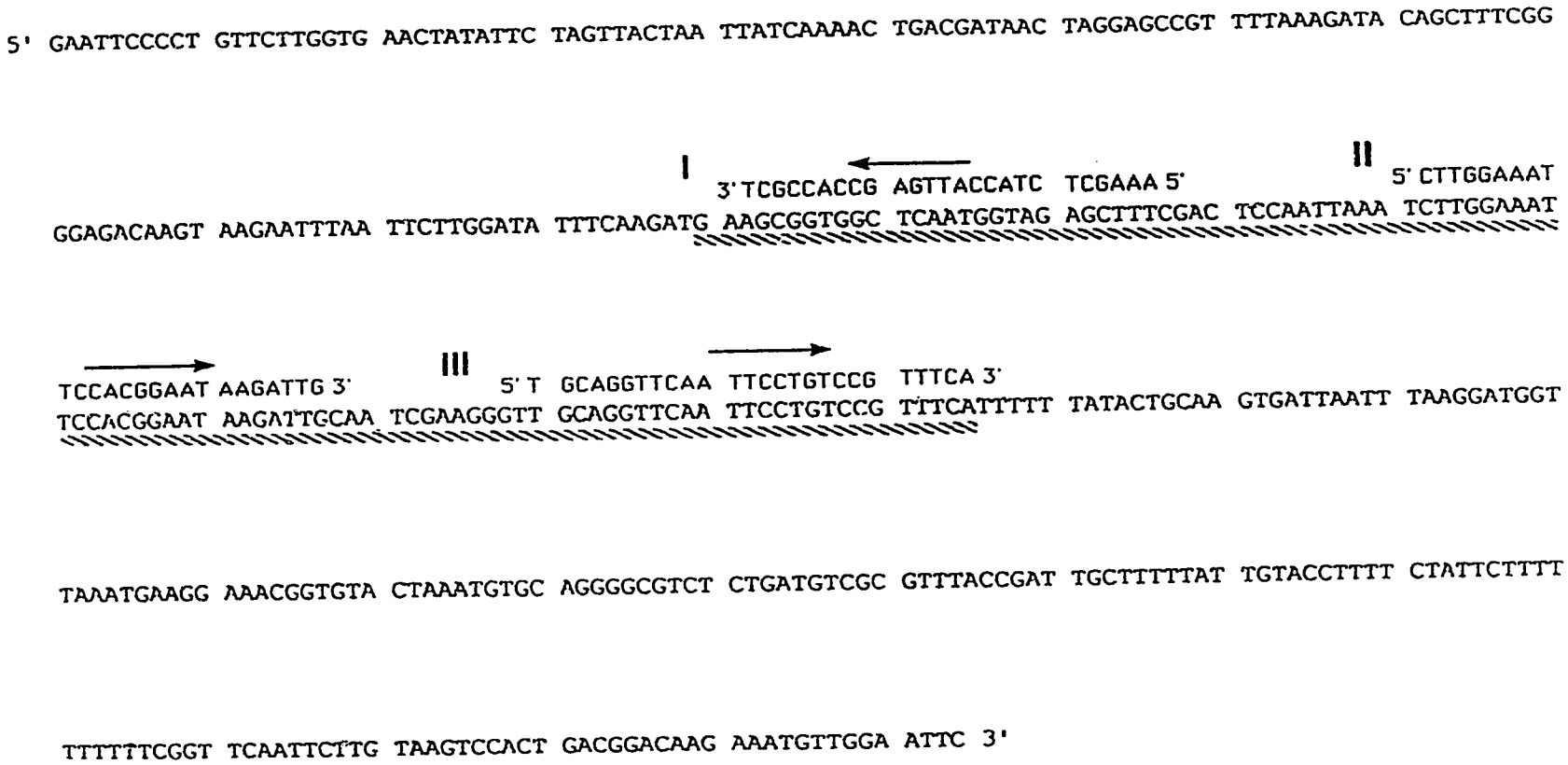


Figure 10: Sequence of a $tRNA^{Trp}$ gene and flanking sequences. The sequence of a 410 bp DNA fragment from *S. cerevisiae* (modified from Kang et al. 1980) is shown, where the $tRNA^{Trp}$ gene is indicated by the stippled line below the sequence. The primers I (ITrp-L), and II (YTrpInt-R) were used for the inverse PCR of the yeast genomic DNA digested with *Sau*3A. The primer III (ITrp-R) was used in inverse PCR experiments as well as a probe to detect the inverse PCR products generated by primers I and II.


5' GAATTCCCCT GTTCTTGGTG AACTATATTC TAGTTACTAA TTATCAAAAC TGACGATAAC TAGGAGCCGT TTTAAAGATA CAGCTTTCGG
I
3' TCGCCACCG AGTTACCATC TCGAAA 5'
II
5' CTTGGAAAT
GGAGACAAGT AAGAATTTAA TTCTTGGATA TTTCAAGATG AAGCGGTGGC TCAATGGTAG AGCTTTCGAC TCCAATTAAA TCTTGGAAAT
III
TCCACGGAAT AAGATTG 3'
5' T GCAGGTTCAA TTCCTGTCCG TTTCA 3'
TCCACGGAAT AAGATTGCAA TCGAAGGGTT GCAGGTTCAA TTCCTGTCCG TTTCATTTTT TATACTGCAA GTGATTAATT TAAGGATGGT
TAAATGAAGG AAACGGTGTA CTAAATGTGC AGGGGCGTCT CTGATGTCGC GTTTACCGAT TGCTTTTTAT TGTACCTTTT CTATTCTTTT
TTTTTTCGGT TCAATTCTTG TAAGTCCACT GACGGACAAG AAATGTTGGA ATTC 3'

Atkin et al. (1992a). The search for the missing sixth gene was investigated by Southern blotting analysis of the original *Sau*3A-digested DNA by hybridizing with the probe used for the Southern analysis of the inverse PCR products (ITrp-R). This Southern blot resulted in 6 bands, with approximately the predicted sizes; 553, 643, 693, 733, 823, and 1233 bp (figure 9A). These sizes represent the sum of the length of the flanking regions of each of the $tRNA^{Trp}$ genes which are amplified using the inverse PCR and the part of the genes which are not amplified using these primers (figure 7). For example, 553 bp represents the sum of the size of ISI (500 bp) and the unamplified part of the related *Sau*3A-digested fragments (53 bp).

Sequencing of the PCR-amplified Flanks

Attempts were made to sequence a number of the fragments that resulted from the inverse PCR experiments (figure 9B). Three of the fragments (IS1, IS2, and IS3) were sequenced after the PCR amplification. The IS2 sequence matched the flanks of the $tRNA^{Trp}$ gene which was already sequenced (Kang et al. 1980). IS1 and IS3 were also sequenced utilizing the primers used for the PCR amplification of these fragments. YTrpInt-R was used to sequence the 3' flanks while ITrp-L was used for sequencing the 5' flanking region of the genes. The sequences of 80-100 bp from each of the 5' and 3' flanking regions of these genes are shown in figure 11. Attempts have been made to clone these fragments into M13mp19 and pUC119, but so far with no success.

Figure 11: The sequences of the 5' and 3' flanking regions of two $tRNA^{Trp}$ genes. Underlined sequences represent the sequences of the 3' end of the genes. The circled bases indicate positions of uncertainty.

IS1 5' flanking sequence

TACTTGTCTG CCGAAATTCT GTGTTTGCTA TAATGTTTGA ATTAAGAATC TCTTAAAATA GCTACTCATA

CTTCTTCATA ACT

IS1 3' flanking sequence

TGTCCGTTTC ATTTTTTTTT TTCCGGAAAA GGGGTCTGGC AAATTTCGCG CGAACGCGTGGAAGTGGGCT

TACTTTCCTT AATTCC

IS3 5' flanking sequence

TCTGTATCTTATGTCTCCCT GTCATCGTCT AAGTCTACTA AATGTTAATT TTAAGTAGGC CTCCTTTGC

CCAAAGTAAG CGCATCATGACGCCCGTCGA

IS3 3' flanking sequence

AATTCCTGTC CGTTTCATTT TCTATTTTCG TGCCCATACC CTTAGCGCCC ACACTCAAATAAAGTCTTTC

GTCTTTCGTG CAAAGGGCGC ACTACGC

DISCUSSION AND CONCLUSION

While informational suppressors in the yeast *S. cerevisiae* have been isolated for $tRNA^{Tyr}$, $tRNA^{Ser}$, $tRNA^{Glu}$, and $tRNA^{Leu}$ genes by genetic techniques (reviewed in Sherman 1982, and Atkin et al. 1992a), no suppressor genes have been mapped to some of the other tRNA gene loci that might be expected to be mutated to a suppressor-encoding gene by a single base pair change to the anticodon region. This includes the yeast $tRNA^{Trp}$ gene family which is the subject of the present study. It has been demonstrated in some cases that the absence of these genetic suppressors is not due to the inability of the altered form of these tRNAs to function as a suppressor. It was first shown by Kim and Johnson (1988) that an amber suppressor of a $tRNA^{Trp}$ gene of yeast can be easily made by oligonucleotide-directed *in vitro* mutagenesis, and this was repeated by Atkin et al. (1990). Other studies showed that *in vitro*-constructed opal suppressor tRNA genes are also functional *in vivo* (Atkin et al. 1990 , and Kim et al. 1990). The *in vitro*-constructed opal suppressor derivative of this tRNA gene acted weakly on the tested opal nonsense mutations (Atkin et al. 1990). It was a surprise that a $tRNA^{Trp}$ gene with a change in the intron as well as in the anticodon was a more efficient suppressor of certain opal nonsense codons (Atkin et al. 1990). In this case it seems that the intron mutation compensates for the initial anticodon mutation, and somehow enhances intron splicing.

There are precedents for base pair changes in tRNA gene

intervening sequences having a deleterious effect on splicing. In the case of *SUP53* it has been shown that some changes to the intervening sequences of this gene result in tRNA secondary structures which are poor substrates for the action of the tRNA splicing endonuclease (Strobel and Abelson 1986b). Other studies have shown that most of the splicing-defective intron mutations have been found to affect the 3' splice site (reviewed in Szekely et al. 1988) where it seems that both the structure and the sequence are important. A single-stranded structure at the 3' splice junction is predicted by lowest free energy calculations for all of the precursors (Ogden et al. 1984). This suggests that the reason for the weak activity of opal suppressors *in vivo* could be an altered secondary structure of pre-tRNA, wherein the 3' splice junction remains double stranded and thus a poor substrate for the action of the tRNA splicing endonuclease (figure 12). Further evidence which supports this suggestion was the accumulation of the precursor of the putative opal suppressor $tRNA^{Trp}$ observed in yeast cells transformed with *in vitro*-constructed opal suppressor $tRNA^{Trp}$ genes with only the requisite anticodon change (Atkin et al. 1990).

This model was extended to encompass amber and ochre $tRNA^{Trp}$ suppressors as well. The amber suppressor $tRNA^{Trp}$ gene product was shown to be efficiently spliced with or without a second change within the intron of the gene. The products of these genes were also shown to be strong amber suppressors when present on single copy plasmids and are toxic when on high copy number plasmids. *In vitro* mutagenesis of the anticodon of a $tRNA^{Trp}$ gene from CCA to TTA did not generate a functional ochre suppressor $tRNA^{Trp}$ *in vivo* (Atkin

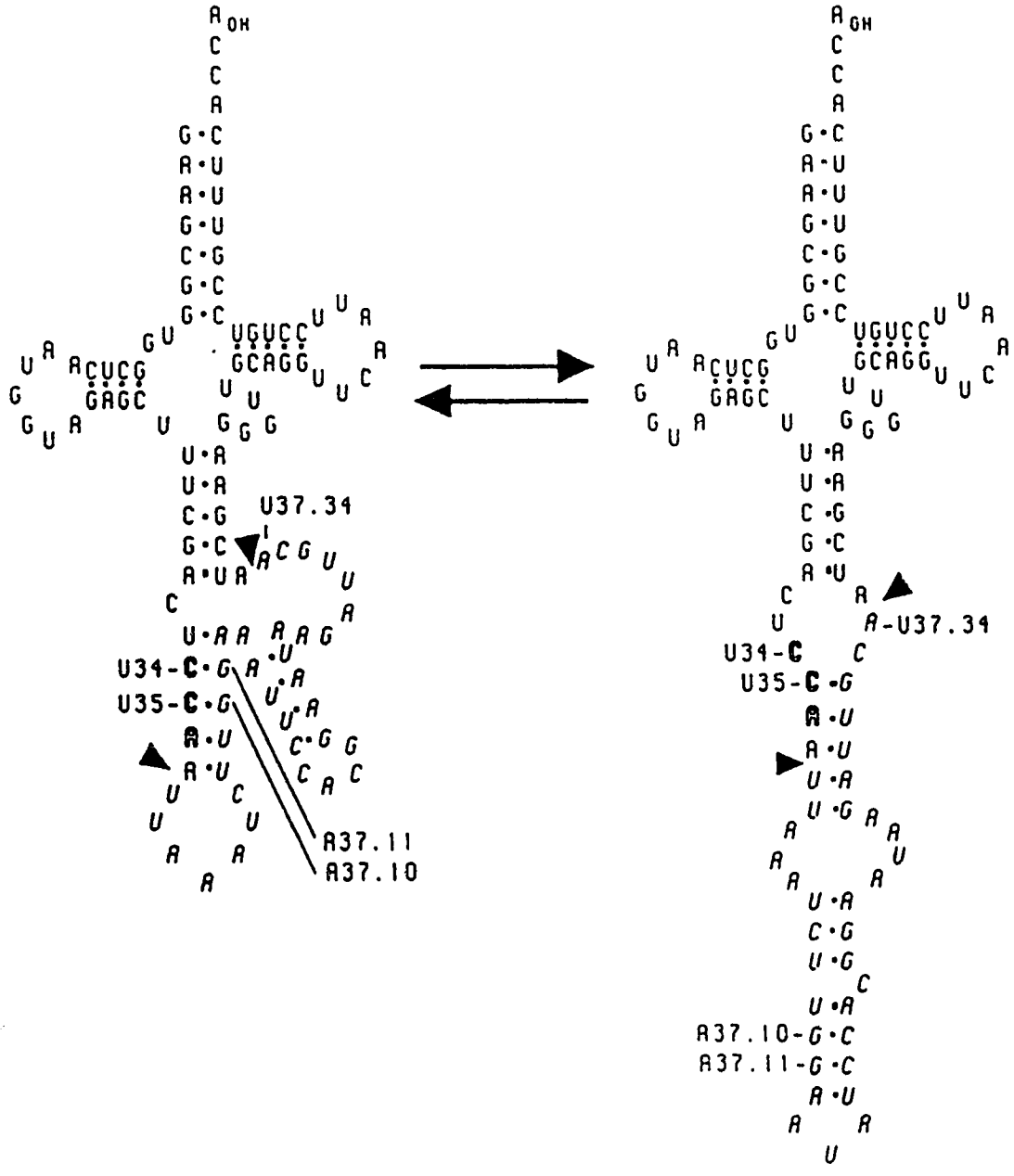
1992), and this was predicted by the model. Moreover, additional changes to the intervening sequences, which supposedly should result in a secondary structure in which the 3' splice junction is again located in a single stranded region, did not result in the production of functional ochre suppressor $tRNA^{Trp}$ (figure 12).

This project was started with the objective of finding if the lack of suppressor ability of these altered $tRNA^{Trp}$ genes could still be a reflection of the inefficient processing of these pre-$tRNA^{Trp}$ transcripts *in vivo*. In order to investigate this, the intervening sequences of the oc1$tRNA^{Trp}$ gene were precisely removed using the appropriate oligonucleotide in a further *in vitro* mutagenesis experiment (figure 1 in the Results section). This resulted in transcripts with no introns and splicing was not necessary. If splicing was the only problem preventing the production of an ochre suppressor, this construct should now produce a functional suppressor. In preliminary experiments, the *in vitro*-constructed intronless ochre suppressor $tRNA^{Trp}$ gene was tested *in vivo* on a single copy plasmid (YCp50) and a multiple copy plasmid (YRp17). While no significant suppression was observed on a low copy number plasmid, there was evidence that transformants containing the intronless construct on a high copy number plasmid displayed weak ochre suppression. Moreover, the weak suppression was seen only when RVB-45C was the host strain. This strain carries an extrachromosomal factor (ψ^+), known to increase the efficiency of ochre suppression (Cox et al. 1988) suggesting that the level of suppression is amplified in a ψ^+ background. The inability of the transformants to grow on ade$^-$ plates proved to be due to the

Figure 12: Possible secondary structures of an unspliced pre-$tRNA^{Trp}$.

The sites on the $tRNA^{Trp}$ gene which were altered by oligonucleotide directed site-specific mutagenesis are shown on the figure. C34 and C35 in the anticodon of this tRNA were substituted with U bases in $oc1tRNA^{Trp}$ construct. The splice sites are indicated by bold arrowheads. Also, the site of the other base substitutions are shown on the figure.

* This figure is reprinted from Atkin 1992.


U37.34
U34-C
U35-C
A37.11
A37.10
A-U37.34
U34-C
U35-C
A37.10-G·C
A37.11-G·C

complete loss of the plasmid when YRp17 was used as the vector. When the cells were replica plated back onto ura⁻ media from YEPD, very slight growth was seen after one week, but only for the transformants using the YCp50-based vectors. This was not surprising; given the mitotic stability of YCp50 plasmids (Struhl et al. 1979).

In subsequent studies, a more stable plasmid, YEp352 was used. This is an episomal plasmid and contains a 2μ circle origin of replication, which results in more mitotic and meiotic stability, as well as remaining in high copy numbers in yeast (Hill et al. 1986). The rationale for utilizing this plasmid as a vector was the fact that if the constructed $tRNA^{Trp}$ gene was, in fact, a weak ochre suppressor the suppression would be strengthened as a result of the continued presence of the suppressor in the cells. Moreover, a variety of host strains harbouring different ochre mutations were utilized. The suppression ability of these tRNA genes was investigated by replica plating the transformants onto omission media, or by a drop test as described in the Results section. In all of the experiments, only the transformants containing the intronless $tRNA^{Trp}$ gene grew on the omission media, indicating that suppression of the ochre mutations occurred only in the presence of the intronless gene. A difference in the suppression efficiency of different ochre mutations, was also observed. The *trp5-48* allele was suppressed when the constructed suppressor gene was present in both high copy numbers and single copy number, with a more efficient suppression when the gene is present on the high copy number plasmid. The other ochre mutations were suppressed only when the suppressor was present in high copy

numbers. However, *lys2-1* was suppressed at a higher efficiency than *lys1-1*, and both were suppressed better than *ade2-1*. That *ade2-1* is a "hard to suppress mutation" is supported by other studies (Reviewed in Hawthorne and Leupold 1974). This is probably due to the context (on mRNA) in which this mutation is located. The ability of the intronless ochre suppressor gene to produce a functional ochre suppressor while the intron-containing $tRNA^{Trp}$ gene did not, suggested that the putative intron-containing ochre suppressor $tRNA^{Trp}$ is somehow unable to be processed *in vivo*. These results strengthen the model of Atkin et al. (1990) that the formation of a secondary structure inappropriate for the action of tRNA endonuclease, probably restricts efficient splicing *in vivo* and as a result affects the efficiency of suppression.

This is the second case reported where the deletion of an intron from a suppressor tRNA gene increases the suppressor ability of a tRNA. The other similar case was observed for a frameshift suppressor $tRNA^{Pro}$ (*SUF8*) where deletion of a 30 bp intron resulted in more efficient suppression compared to the intron-containing *SUF8*, apparently because of relatively impaired splicing of the intron-containing *SUF8* pre-$tRNA^{Pro}$ (Winey et al. 1986). In contrast, removing the intron from *SUP61-a* and *SUP61-o* (serine-inserting amber and ochre suppressor $tRNA^{Ser}$ genes, respectively) was found to cause no change in the ability of the tRNAs to suppress certain mutations, or in the level of mature $tRNA^{Ser}$ transcribed *in vivo* (Ho and Abelson 1988). This suggests that the introns of many tRNA genes are dispensable for the gross functioning of the genes. Thus, these introns may be considered to be redundant. However, other

studies have demonstrated that introns may play an important role in gene function for some yeast tRNA genes (Johnson and Abelson 1983, Strobel and Abelson 1986a). These studies identified less efficient suppression, and a lack of a nucleoside modification normally present in the anticodon of the *SUP6-o* and *SUP53-a* tRNAs, when the cognate intron was deleted from these tRNA genes. These results suggest that the intron of the ochre suppressor $tRNA^{Trp}$ in this study may also be involved in the modification of the 5' U in the anticodon. In *Saccharomyces cerevisiae*, ochre (UAA) suppressor tRNAs are allele specific, supposedly due to the modification of uridine to 5-methoxycarbonyl-methyl-2-thiouridine at the wobble position of the anticodon (Laten et al. 1983). The allele specificity of the putative ochre suppressor from the intronless gene was tested by transforming yeast cells harbouring the amber nonsense mutations, *trp1-1*, and *met8-1*. After plating them on omission media, no growth was seen, even after incubation at 30°C for 12 days. This result suggested that if the U base in the anticodon of this tRNA is modified, the intervening sequence in the pre-tRNA from this gene is not involved in the modification. In other words, modification, if it happens, may happen after the removal of the intron in the pathway of the processing of this pre-tRNA. Lack of suppression could also be due to the insertion of an inappropriate amino acid at the position of these amber mutations by the constructed $tRNA^{Trp}$, which results in a nonfunctional protein. We have no evidence whether the constructed tRNA gene codes for a tRNA which is aminoacylated with a tryptophan AAS or with another AAS. Alternatively, since the ochre suppression observed herein was very weak even with the

oc1ΔIVStRNA^{Trp} gene. any affect on amber suppression may be below the sensitivity threshold for the assay used in this experiment. Further studies, beyond the present investigation, would be needed to resolve this problem.

Another interesting aspect of this work was the significant enhancement of ochre suppression by the intron-deleted construct when an extrachromosomal element, ψ^+, was present. The ψ^+ factor is known to increase the ochre suppression ability of some ochre suppressors and certain frameshift suppressors (reviewed in Cox et al. 1988). The effect of the ψ^+ factor was shown by transforming a ψ^+ (RVB-45C) and a ψ^- (RVB-4B) strain with the *in vitro*-constructed oc1ΔIVStRNA^{Trp} gene, and then assaying the suppression efficiency of an ochre mutation present in both of the strains (*lys1-1*). Almost two orders of magnitude difference in growth was observed between these two strains when different concentrations of the cells were plated on a ura$^-$ lys$^-$ media (figure 5). That the suppressor-enhancing effect was specifically due to ψ^+ was shown by eliminating this factor from RVB-45C cells which were transformed with oc1ΔIVStRNA^{Trp} gene in media where continued suppression was necessary for growth. The elimination of the ψ^+ factor in hypertonic media has been demonstrated by Singh et al. (1979). After incubating these cells in ura$^-$ lys$^-$ media containing 0 M, 1 M, or 2 M KCl, a long delay was seen in the growth of the cells in 1 M KCl media and the doubling time was significantly extended compared to growth with no KCl. Moreover, no growth was observed for the cells in the 2 M KCl media. The possible inhibitory effect of the hypertonic media on

yeast cell growth in general was monitored by growing a prototrophic strain (JG113-5R: ura$^+$ lys$^+$) in identical hypertonic media. Almost no difference was seen in the growth rate of these cells in 0 M and 1 M KCl ura$^-$ lys$^-$ media. Despite a two-day delay in the 2 M KCl containing ura$^-$ lys$^-$ media, these control cells eventually grew well, although the doubling time was extended. This was clear evidence that the ochre suppression, which was observed in RVB-45C cells transformed with oc1ΔIVStRNA^{Trp} gene, was due to the continued presence of the ψ^+ factor in this strain. This is the first case reported where the ψ^+ factor can enhance ochre suppression directed by an *in vitro*-constructed tRNA suppressor. The nature of this extrachromosomal factor and how it helps suppression of ochre nonsense mutations is still unknown. However, as reviewed in Cox et al. (1988), ψ^+ could be a repressor of a factor present in yeast cells which enhances the fidelity of translation, particularly chain termination. Therefore, an ochre suppressor tRNA, which is competing with the natural termination mechanisms for the cognate termination codon at the ribosomal A site, has a better chance to read through the cognate mRNA when the ψ^+ factor is present. On the other hand, ψ^+ could be an altered form of one of the components of the translational termination complex. Many alterations in protein-coding genes such as missense mutations in λ cro, N terminal region of λ repressor, or yeast iso-1-cytochrome C, have been shown to cause temperature sensitivity. Presumably, this is because of the increased ratio of the unfolded or misfolded to properly folded form of these proteins in cells at the restrictive temperature (reviewed in Pakula and Sauer 1989). In the present study no relationship was

observed between the suppressor ability of the constructed tRNA^{Trp} gene in a ψ^+ background at room temperature versus a higher temperature (37°C). This could be interpreted that if ψ^+, in fact, codes for an altered form of a protein present in a ψ^- cell, the alteration does not cause a heat sensitivity. This line of experimentation could be extended in order to investigate the nature of ψ. With the results reported herein, we now have a system which can differentiate between ψ^+ and ψ^- cells phenotypically, by affecting the efficiency of suppression of certain ochre mutations in the presence of the *in vitro*-constructed intronless ochre suppressor tRNA^{Trp} gene. Therefore, the physical identity of the element may be determined by extracting various fractions of ψ^+ cells and cotransforming ψ^- yeast cells with the extracted fraction and the intronless tRNA^{Trp} gene, and then trying to rescue the ochre suppressor ability phenotype. Further investigations on how ψ affects the translation termination mechanism can also be done with an *in vitro* translation assay, where the rate of readthrough of an ochre nonsense mutation can be assayed in the presence or absence of the ψ^+ factor.

Moreover, to further demonstrate the inability of the putative intron-containing ochre suppressor tRNA^{Trp} gene to act as a functional suppressor, transcript analysis should be done to demonstrate that an insufficient amount of mature ochre suppressor tRNA^{Trp} accumulates in cells transformed with this gene. In addition, a solution-structure probing (Szekely et al. 1988) could be done to actually identify the secondary structure of the putative ochre suppressor tRNA^{Trp}, and identify the stage in splicing of the intron of

the gene which is defective.

In the second part of this work, the 5' and 3' flanking regions of the six $tRNA^{Trp}$ genes of *S. cerevisiae* were studied. While the promoter function in tRNA genes has been attributed to sequences inside the genes (Ciliberto et al. 1982), *in vitro* and *in vivo* studies have demonstrated the involvement of the 5' and 3' flanking regions in the transcription of some tRNA genes (Shaw and Olson 1984, Marshalek and Dingermann 1988, and Allison and Hall 1985). The number of $tRNA^{Trp}$ genes in the haploid genome of *S. cerevisiae* has been determined to be six (Atkin et al. 1992a). Utilizing primers that were constructed on the basis of the sequence of one of the $tRNA^{Trp}$ genes, DNAs corresponding to the other members of this tRNA gene family were amplified and sequenced (Atkin et al. 1992a). The sequences of the coding regions and the 34 bp intron of these genes are identical. This fact was utilized herein for amplification of the flanking regions of these genes, with an inverse PCR methodology (figure 7). Yeast genomic DNA was digested with different restriction enzymes, religated and amplified using the outward-directed primers complementary to both ends of the tRNA genes (figure 7). Only the *Sau*3A-digested DNA resulted in bands with appropriate sizes for easy sequencing. The primers (ITrp-L and YTrpInt-R) were chosen so that part of the genes were amplified along with the flanks (figure 11). Another primer (ITrp-R) complementary to the amplified part of the gene was used as a probe in Southern hybridization analyses to demonstrate that the true flanking DNA fragments were amplified (figure 9). Consequently, this technique yielded five DNA

fragments from PCR amplification (IS1-IS5, figure 9B) which are authentic 5' and 3' flanking regions of five of the six tRNA genes present in the yeast *Saccharomyces cerevisiae* (Atkin et al. 1992a). Moreover, two faint bands were also observed on ethidium bromide stained gel (IS6 and IS7, figure 9B). Southern analysis of yeast genomic DNA digested with *Sau*3A demonstrate that IS7 is a true amplification of a $tRNA^{Trp}$ gene and its flanks. The low yield of this fragment when amplified using PCR technology could be because of the larger size of this fragment. This could cause less self-ligation of the fragment, and as a result less inverse PCR products compared to the other fragments. IS6 did not hybridize to the probe in the genomic Southern hybridization analyses, meaning that it is possibly due to a nonspecific binding of the PCR primers. Attempts to reamplify the biggest fragment (IS7) by cutting the band out of the gel, and reamplifying it with PCR failed. This failure was possibly because of the lower yield of this fragment which resulted from the original inverse PCR (figure 9B in Results section). Therefore, it is possible that the template DNA which was extracted from the gel using a Geneclean kit was not enough for a successful PCR reaction. Three of the recovered fragments were sequenced. IS2 was sequenced directly from the PCR products and proved to be the flanks of the $tRNA^{Trp}$ gene which had already been sequenced (Kang et al. 1980, figure 10). The sequencing of IS1 and IS3 was rather challenging. Several attempts to clone these fragments into M13mp19 or pUC119 *Hin*cII and/or *Sma*1 sites failed. Finally, these fragments were sequenced directly after PCR amplification. Direct sequencing of short double stranded DNA products from PCR

reactions has proven to be a difficult task, due to the tendency of the templates to reanneal (Bachmann et al. 1990). The protocol used for the sequencing of the PCR fragments (see Materials and Methods) did not produce sequences further than 80-100 bp from the 5' and 3' ends, in my hands. Moreover, the strong background made the sequences difficult to interpret accurately. Various studies have demonstrated that sequences not further than 70 bp at the 5' and 3' side of the tRNA coding regions are involved in transcription (Sajjadi and Spiegelman 1989, Shaw and Olson 1984, and Allison and Hall 1985). Thus, if the sequences of the flanking regions of these $tRNA^{Trp}$ genes are also important in transcription, it is most likely inside the sequenced region. The sequences of the 5' and 3' flanking regions of IS1 and IS3 are shown in figure 11 in the Results section. YTrpInt-R was used to sequence the 3' flanks while ITrp-L was utilized for sequencing the 5' flanks. The sequences of the 5' flanking regions of these three $tRNA^{Trp}$ genes show no conserved sequences, beyond a general abundance of A and T residues. However, the 3' flanking sequences of all of these genes start with a short T array which is presumably the site of transcription termination. No other conserved sequence is observed in the flanking regions of these genes. The lack of sequence conservation within the flanking regions of the $tRNA^{Trp}$ genes which are sequenced herein, could denote different transcription efficiencies among individual members of this tRNA gene family, where transcription is regulated by positively or negatively regulating elements located upstream or downstream of these genes.

For further investigations of the 5' and 3' flanking regions of this

tRNA gene family, the other $tRNA^{Trp}$ gene flanks should be sequenced. The next step will be constructing primers on the basis of the sequences of the flanks of each $tRNA^{Trp}$ gene. Thus, these primers can be used to target each specific $tRNA^{Trp}$ gene of *Saccharomyces cerevisiae* back to its native chromosomal context. This could obviously include *in vitro* altered versions of any of these genes. Once the genes and their flanks are cloned, we can investigate the expression of individual $tRNA^{Trp}$ genes through assaying suppressor ability of the putative *in vitro*-constructed nonsense suppressor of the genes. Also, the involvement of the 5' and 3' flanking regions of each gene in the transcription of the gene can be assayed by deleting the flanks to different extents, and then investigating the amount of transcription *in vitro* by an *in vitro* transcription assay, or *in vivo* by looking at the suppressor ability of the 5' and/or 3' deleted suppressor $tRNA^{Trp}$ genes. The 5' and 3' flanking sequences could also be utilized for construction of different primers which enable us to continue disruption experiments of the $tRNA^{Trp}$ genes by targetted insertion, in order to find the minimal number of these genes which are required in *S. cerevisiae*. This will give us a hint as to why no natural $tRNA^{Trp}$ suppressors have been found using conventional genetic methods. Finally, we now have the ability to compare the *in vivo* suppression ability of an introduced suppressor gene residing on a plasmid to the same suppressor construct when it replaces a specific wild type member of the $tRNA^{Trp}$ gene family. Therefore, transcription of each $tRNA^{Trp}$ gene at the native chromosomal location can be investigated.

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