STRUCTURAL FEATURES OF THE 5' ETS IN *Schizosaccharomyces pombe* ESSENTIAL FOR RIBOSOMAL RNA MATURATION

A Thesis

Presented to

The Faculty of Graduate Studies

of

The University of Guelph

by

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In partial fulfilment of requirements

for the degree of

Doctor of Philosophy

February, 1999

ABSTRACT

STRUCTURAL FEATURES OF THE 5' ETS IN *Schizosaccharomyces pombe* ESSENTIAL FOR RIBOSOMAL RNA MATURATION

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The 5' external transcribed spacer (5'ETS) of genes encoding ribosomal RNAs (rRNA) in *Schizosaccharomyces pombe* was examined with respect to structural features which underlie rRNA maturation. These studies utilized a previously reported experimental system with which a cloned rDNA repeat that was "tagged" in the 5.8S rRNA gene can be manipulated *in vitro* before being efficiently expressed in *S. pombe*. This system was further improved during the course of this work with the introduction of a functionally neutral "tag" into the 18S ribosomal DNA so the maturation of the 18S rRNA could be detected differentially by hybridization analysis. Initial attempts to perform a mutational survey of this spacer by PCR-based mutagenesis strategies were unsuccessful, and led to the development of two new PCR-based methods. Subsequently, the results of the survey indicated that the first and last of the seventeen helical domains were virtually dispensable but in contrast the internal domains were shown to be critical for 18S rRNA maturation. In contrast to previous reports in *Saccharomyces cerevisiae,* computer analyses and partial digestion with nuclease probes of the region most proximal to the 18S rRNA indicated a crucifix-like structure composed primarily of three extended hairpins. A reevaluation of the same region in *S. cerevisiae* indicated a conserved core structure, including the U3 snoRNA binding site within this higher order structure. Based on the new structural estimates, smaller

changes were systematically introduced into the critical regions and strong correlations were observed with known or putative events in rRNA maturation. Changes associated with an intermediate cleavage site in helix II, the end of loop III and with the putative U3 snoRNA binding site again were, critical to 18S rRNA production. Further analyses of the 5.8S rRNA indicated that the large ribosomal subunit RNA can be properly processed in most cases but the efficiency is significantly reduced, an observation which provides new evidence of interdependency in the maturation process. The results illustrate that rRNA processing is more critically dependant on the 5'ETS than previously believed and also give further evidence that the primary transcript may be processed in one large complex.

ACKNOWLEDGMENTS

I would like to thank Dr. Ross Nazar for taking a chance on an individual who had been out of the field for seven years and also for his invaluable supervision and "beatings". I also want to thank my advisory committee: Dr. Alan Wildeman and Dr. Jane Robb for helpful discussions and constructive criticisms.

I am indebted to my wife, Nikki, and to Yvonne Boers for all of their help in preparing this document. The following present and former lab members have been particularly helpful in sharing ideas and laughter : Phil Shwed, Zhenya Ivakine, Dr. Liam Good, Dr. Bill Wong, Dr. Atanas Lalev and Sean Song. I also appreciate other members of the department for their discussions and assistance.

This thesis is dedicated to, Nikki, whose love and continuing support made the last four years enjoyable and also to my girls: Samantha, Victoria and Jody who constantly remind me that you must walk before you can run.

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^{32}P	radioactive isotope of phosphorus (half life, 14.3 days)
A_{260nm}	absorbance at 260 nanometers
Amp, Amp^r	ampicillin, ampicillin resistance gene
ATP	adenosine triphosphate
bp	base pair
BSA	Bovine serum albumin
°C	degrees Celsius
CA	California
$CaCl_2$	calcium chloride
cDNA	complementary DNA
Ci	Curies
cm.	centimetre
CPBF	core promoter binding factor
CPE	core promoter element
CsCl	Cesium chloride
C-terminal	carboxy terminal
Ctl	control
dATP	2'-deoxyadenosine 5'-triphosphate
Dbp 7p	putative helicase
dCTP	2'-deoxycytosine 5'-triphosphate
ddH_20	double distilled water

ddNTP	2'3'-dideoxynucleoside 5'-triphosphate
DFC	Dense Fibrillar Component
dGTP	2'-deoxyguanosine 5'-triphosphate
Dim 1p	18S rRNA dimethylase protein
DMSO	Dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	2'-deoxynucleoside 5'-triphosphate
DOB 1	gene encoding a putative helicase
DRS 1	gene encoding a putative helicase
dsDNA	double strand DNA
DTT	dithiothreotol
dTTP	2'-deoxythymidine 5'-triphosphate
E coli.	*Escherichia coli*
EDTA	ethylene diamine tetracetic acid
E.M.	electron microscopy
EtBr	ethidium bromide
EtOH	ethanol
ETS	external transcribed spacer
Fal 1p	putative helicase
FC	Fibrillar Centers
g	gram
GAR 1	gene encoding the Gar 1p protein

GC	Granular Component
GTPase	2'-deoxyguanosine 5'-triphosphate hydrolase protein
HCl	hydrochloric acid
H_2O	water
hr	hour
ITS	internal transcribed spacer
ITS1	internal transcribed spacer 1
ITS2	internal transcribed spacer 2
IVS	intervening sequences
KAce	potassium acetate
kb	kilobase
KCl	potassium chloride
Ku/1BF	protein component of polymerase 1 transcription complex
LB	Luria-Bertanini bacterial growth media
LiCl	lithium chloride
LSU	large subunit
L1	large subunit ribosomal protein
L16	large subunit ribosomal protein
L29	large subunit ribosomal protein
M	molarity
mA	milliamps
MA	Massachusetts

MD	Maryland
mg	milligram
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulfate
min.	minute
ml	milliliter
mm	millimetre
mM	millimolar
MMLV	Moloney murine leukemia virus
$MnCl_2$	Manganese chloride
MO	Missouri
mRNA	messenger ribonucleic acid
mTTF	mouse polymerase 1 termination factor
N	normal
Na	sodium
NaAce	sodium acetate
Na-Citrate	sodium citrate
NaCl	sodium chloride
NaOAce	sodium acetate
NaOH	sodium hydroxide
ng	nanogram
N.J.	New Jersey

nm	nanometer
NOP 1	gene encoding a protein involved in ribosome biogenesis
NOP 5	gene encoding a protein involved in ribosome biogenesis
nt	nucleotide
NTS	non-transcribed spacer regions
NY	New York
$OD_{550\ nm}$	optical density taken at 550nm
OK	Oklahoma
Ori(e)	*E. coli.* origin of replication
Ori(p)	*S. pombe.* origin of replication
PAC 1	gene encoding a protein involved in ribosome biogenesis
PCR	Polymerase Chain Reaction
P.d.	plasmid-derived
PEP	plasmid enhanced PCR-mutagenesis
pH	concentration of hydrogen ions
pmol	picamol
pol	polymerase
Pop1p	protein component of RNase MRP
psi	pseuduridine
RAT 1	gene encoding a nuclease involved in ribosome biogenesis
Rb ^{110}	Retinoblastoma protein
rDNA	ribosomal DNA

Reb 1p	protein involved in pol I termination in *S. cerevisiae*
rib 1	protein involved in pol I termination in *Xenopus laevis*
RNA	ribonucleic acid
RNP	ribonucleoprotein complex
RNT 1	gene encoding an endonuclease thought to perform the A1 and D1 cleavages
rpm	revolutions per minute
r-proteins	ribosomal proteins
rRNA	ribosomal RNA
RNase P	RNA component of RNase MRP complex
Rok 1p	putative helicase thought to be involved in rRNA maturation
Rrp 3p	putative helicase thought to be involved in rRNA maturation
RRP 4	ribosomal protein thought to be involved in rRNA maturation
S. cerevisiae	*Saccharomyces cerevisiae*
SDS	sodium dodecyl sulphate
SL1	protein component of transcription initiation complex
SOF 1	trans-acting factor involved in rRNA processing
snoRNAs	small nucleolar RNAs
snoRNP	small nucleolar ribonucleoprotein complexes
snR10	trans-acting factor involved in rRNA processing
snR30	trans-acting factor involved in rRNA processing
S. pombe	*Schizosaccharomyces pombe*
SpReb 1p	*S. pombe* pol I termination factor

SSOE	single strand overlap extension
SSU	small subunit
Taq	DNA polymerase enzyme from *Thermus Aquaticus*
TB	Terrific broth
TBP	TATA binding protein
TBP_I	TATA binding complex associated with pol I
Tet^r	tetracycline resistance gene
tis	transcription initiation site
TLC	thin layer chromatography
tRNA	transfer ribonucleic acid
UBF	upstream binding factor
UCE	upstream control element
uM	micromolar
Ura	uracil
ura4-D18	*S. cerevisiae* URA 3 gene
U.V	ultraviolet light
U3 RNA	RNA component of U3 snoRNP
U8 snoRNP	transacting factor involved in 3' end maturation in *Xenopus*
U14 sno RNA	transacting factor involved in 5' ETS processing
U22	transacting factor involved in 5' ETS processing
V	volts
v/v	volume per volume

W	Watts
W.t.	wild type
w/v	weight per volume
XRN 1	exonuclease thought to be involved in rRNA maturation

Chapter 1 Introduction

A. Ribosome overview

Ribosomes are large ribonucleoprotein complexes that are essential for protein synthesis. They first were identified by Claude, (1938, cited in Tissieres, 1974) fifty years ago during electron microscopy studies on Rous Sarcoma virus infected cells and at the time were termed microsomes. It was unknown if the presence of microsomes was a result of viral infection, or if they were normal cellular organelles. In subsequent studies, Claude, (1941, cited in Tissieres, 1974) and others (Jeener and Brachet, 1941, cited in Tissieres, 1974) isolated microsomes from various uninfected tissues and showed that they always contained ribonucleic acid (RNA). At this time, it was first hypothesized that microsomes were an essential component for protein synthesis (Jeener and Brachet, 1942, cited in Tissieres, 1974).

Evidence for a role in protein synthesis was first presented when Borsook et al., (1950, cited in Tissieres, 1974) observed that 30 minutes after injection of labeled amino acids into mice, the microsomes had the highest specific radioactivity of the various cellular fractions and similar results were obtained by Hultin, (1950, cited in Tissieres, 1974). A clear demonstration that microsomes were the sites of protein synthesis came in 1955. Littlefield et al. injected rats with radioactive amino acids, isolated microsomes from liver cells and were able to show that within minutes the microsomes again had the highest specific radioactivity associated with them, however, the microsomal proteins themselves labeled much more slowly. These studies were later confirmed *in vitro* utilizing cell free extracts and pulse chase experiments (Littlefield and Keller, 1957, cited in Tissieres, 1974).

At the same time as this work was being performed, it also was noted that the microsome particles consisted of approximately 50% protein and 50% RNA. To reflect the high RNA content of microsomes, Roberts, (1958, cited in Tissieres, 1974) renamed them "ribosomes". The general features of ribosome morphology and it's function as the translational apparatus are fully accepted (to date), but it's evolution, biogenesis, enzymology and finer morphological features are subjects which are still debated widely.

Ribosomes are composed of two unequal subunits termed: the small subunit (SSU) and the large subunit (LSU) and this architecture has been conserved throughout the kingdoms (Lake, 1981, 1985). In prokaryotes, the two subunits have sedimentation coefficients of 30S and 50S whereas the eukaryotic counterpart values are 40S and 60S (Brimacombe et al., 1978, Sommerville, 1986). The size differences between the two come from differences in both the protein and rRNA composition. The prokaryotic LSU consists of the 5S and 23S ribosomal RNAs (rRNA) and 34 ribosomal proteins (r-proteins) and the SSU contains a 16S rRNA and 21 r-proteins associated with it (Wittman, 1983). Like the prokaryotic SSU, the eukaryotic SSU contains one rRNA species, the 17-18S rRNA, however, there are approximately 32 r-proteins. The eukaryotic LSU also contains a greater number of r-proteins (42-43) (Verschoor et al., 1998) than its prokaryotic counterpart, as well as the 25-28S, 5S and 5.8S rRNA species (Sommerville, 1986). While the eukaryotic LSU would appear to have a greater number of rRNA species, the 5.8S rRNA was demonstrated to be homologous with the 5' end of the 23S prokaryotic rRNA and is hydrogen bonded with the 5' end of the 25S rRNA (Nazar, 1980). Further differences lie in the protein to rRNA ratio. Prokaryotic ribosomes consist of approximately 37% protein

and 63% rRNA whereas the ratio in eukaryotic ribosomes appears to be equal between the two components (Wittman, 1983, Verschoor, 1998). The reasons for the differences between the kingdoms are unclear but it has been postulated that the prokaryotic ribosome has been streamlined to allow rapid growth and/or the eukaryotic ribosome may be more complex for regulation reasons (Moldave, 1985, Noller, 1991).

With overall sizes that range from 25-35 nm, ribosomes are large enough to be visualized by the electron microscope, which is used extensively to study the three dimensional structure and association of the subunits. These studies have revealed that all ribosomes are strikingly similar (Lake and Kahan, 1975, Shatsky et al., 1980, Stoffler and Stoffler-Meilicke, 1984, Lake, 1985). The SSUs appear embryo shaped, with a head and body connected by a narrow neck. The neck is formed by a notch on one side and a deep cleft on the other and a slight bending of the SSU produces a flattened region on one side called the platform. The eukaryotic SSU has additional distinguishable morphological features that include: an angled ear and bill that extends from the head and lobes at the base of the body (Verschoor et al., 1996). However, more recent studies that have allowed better resolution show that the prokaryotic SSU has a rudimentary beak as well (Verschoor et al., 1998). The LSUs appear to be boat shaped with the more pointed end of the boat marked by a central protuberance and stalk. Yonath and Wittman, (1989) also were able to resolve a tunnel 10 nm long and 2.5 nm wide in the large subunit and it has been postulated that the nascent polypeptide passes through this tunnel as it is being made. Again, the eukaryotic LSU has additional features, the most notable being two horizontal extensions which look like a fork (Verschoor et al., 1996) at the base of the stalk . The two subunits come together

in the cytoplasm at the time of protein synthesis when the platform of the SSU contacts a groove in the LSU. The SSU holds the messenger RNA (mRNA) and the LSU catalyzes the formation of the peptide bonds as the mRNA passes through the complex (reviewed by Moldave, 1985 and Noller, 1991).

Protein synthesis is an essential process for all living cells. The eukaryotic cell must be able to regulate both the total amount of specific proteins as well as the total protein produced in response to environmental stimuli, changes in growth conditions or stage of the cell cycle. The protein synthetic capacity of a cell is regulated by a number of factors such as the availability of mRNA, efficiency of translation, availability of translation factors and the number of ribosomes (Hannan et al., 1998). To date, the accumulated evidence suggests that the cell's ability to synthesize proteins is regulated primarily by the steady state number of ribosomes (reviewed in Paule, 1994). In the majority of cells, ribosomes are relatively stable therefore the total number in the cell is dependent on the rate of their biogenesis.

As the growth rate of the cell is altered the rate of synthesis of the ribosomal components can be altered by as much as three orders of magnitude. In order to maintain a balance of the four rRNAs and the 75 ribosomal proteins the cell must coordinate the: 1) regulation of the three transcriptional systems mediated by RNA polymerases I, II, and III, 2) RNA processing, 3) export of the r-protein mRNAs to the cytoplasm, 4) their translation and import back into the nucleus, 5) the gathering of all the components in the nucleolus, 6) formation of the ribosomal subunits and 7) finally their transport back into the cytoplasm. Experimental evidence so far has correlated the regulation of ribosome biogenesis with altered rates of RNA polymerase I transcription versus rRNA stability or processing

(reviewed by Moss and Stefanovsky, 1995). To explain these correlations a model has been proposed in which non-translating ribosomes either directly or indirectly repress rRNA synthesis (Nomura et al., 1984). Therefore, it would appear that the biogenesis of ribosomes is controlled through a feedback regulation mechanism of rDNA transcription.

B. rDNA organization in eukaryotic nucleoli.

The hundreds or thousands of rRNA gene repeats, which encode the ribosomal RNAs of cytoplasmic ribosomes, are clustered at sites on one or several chromosomes in eukaryotic genomes. During mitosis, these clusters are seen as secondary constrictions and in interphase cells they are identified as nucleolar organizers. In general, there is a correlation between the number of rRNA genes and an evolutionary increase in the DNA content per haploid genome (Mandal, 1984). Prokaryotes contain as many as twelve copies of rDNA and lower eukaryotes have between 100 and 200 copies, whereas higher plants have as many as 30,000 copies of rDNA (Ingles et al., 1975). However, large variations in rDNA copies can be seen not only between species but between individuals of a species (Long and Dawid, 1980). For example, amphibians, bony fishes and some insects have the ability to amplify their rDNA content during oogenesis and the newly replicated copies exist extra chromosomally (Brown and Spence ,1968, Chaudhuri and Mandal, 1980, Gall and Rochaix, 1974). Such amplifications result in significant differences between individuals. Also, in *Tetrahymena pyriformis* there is only one rDNA copy in the genome and the rest are extra chromosomal (Karrer and Gall, 1976).

All rRNA genes are organized in repeating pre-rRNA transcription units separated by non-transcribed spacer regions (NTS) (Figure 1). The pre-rRNA transcription unit

consists of : the mature rRNA sequences (17/18S, 5.8S, 25/28S), external transcribed spacers that flank the mature sequences (5' ETS and 3' ETS) and internal transcribed spacers that are located between the mature sequences (ITS1 and ITS2). The polarity of transcription is always 5' ETS-(17/18S)-ITS1-(5.8S)-ITS2-(25/28S)- 3' ETS, a polarity which is universal even in prokaryotic (Nomura et al., 1977) and organellar (Whitfeld and Bottomley, 1983) rRNA genes.

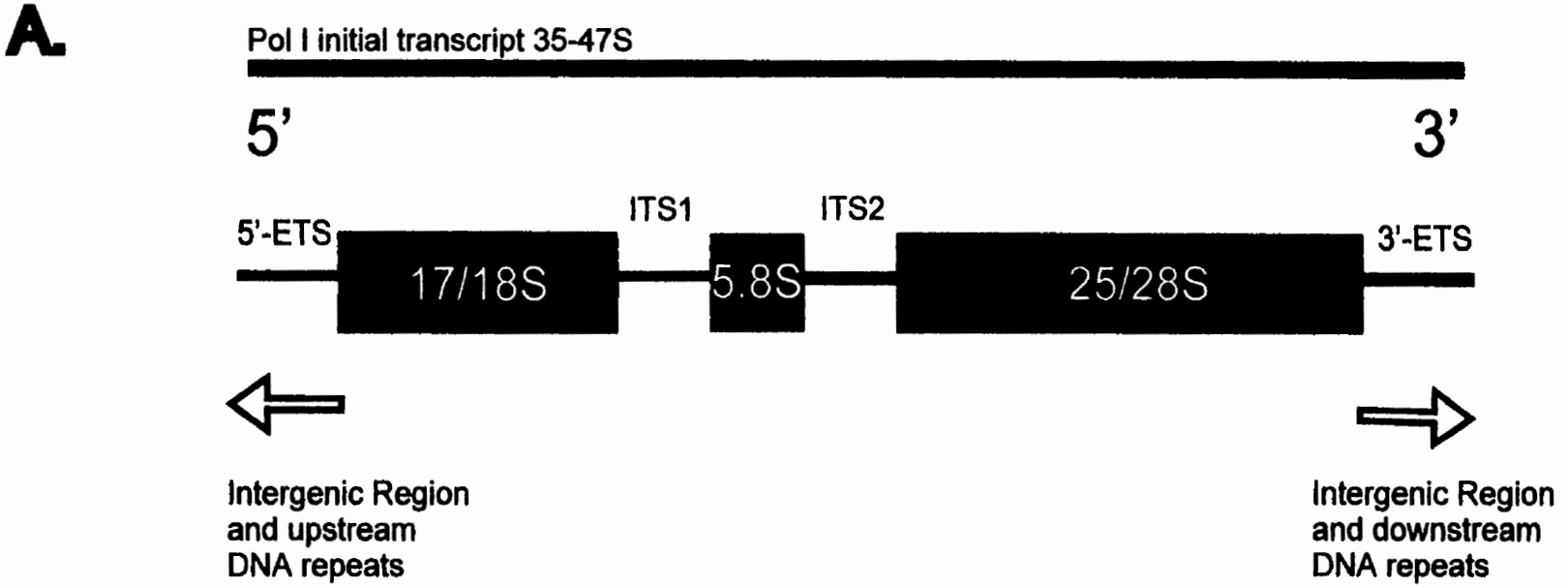
Although the organization of the rRNA genes has remained stable throughout evolution, there are large variations in size of the eukaryotic transcript, with yeasts being the smallest (7kb) and mammals the largest (13kb) (Klootwijk et al., 1979, Wellauer and Dawid, 1974). These differences are due to expansion segments (discussed later) within the mature genes as well as increased transcribed spacer lengths . The non-transcribed spacer lengths also vary considerably in the different organisms and, as a result, can add to differences in the rDNA repeat unit (primary transcription unit and NTS).Yeast for example has a 10 kb repeat length while mammals are 40 kb in total length (Klootwijk et al., 1979, Cory and Adams, 1977).

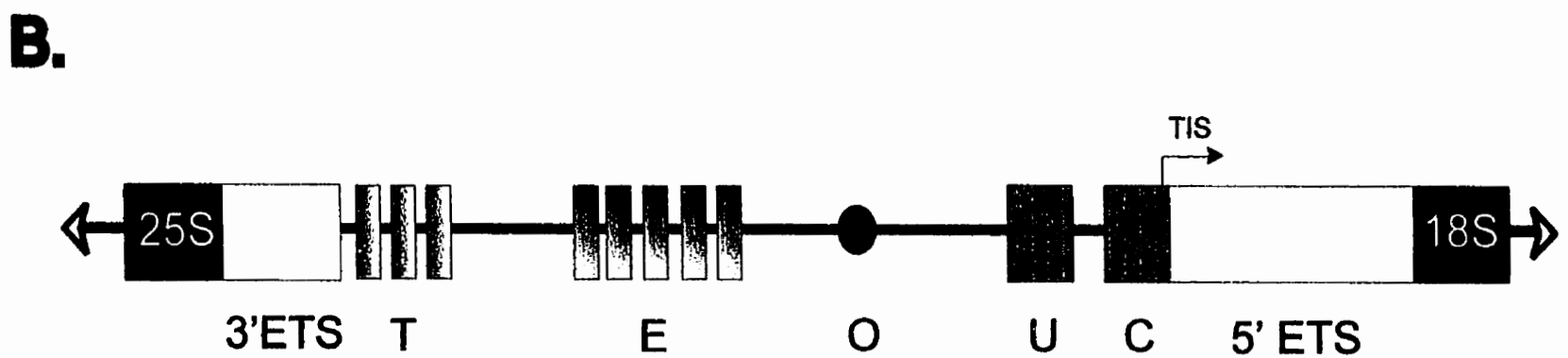
Intervening sequences (IVS) or ribosomal insertions within the mature rRNAs sequences are atypical, however, they are present in some dipteran insects and lower eukaryotes (Barnett and Rae, 1979, Cech and Rio, 1979). Approximately 50 percent of *Drosophila melanogaster* rRNA genes have inserted sequences, which vary in size from 0.5 to 5 kb, within the 28S rRNA gene sequence (White and Hogness, 1977). These sequences are unable to be processed from the transcript and therefore the repeats where they are present represent rRNA pseudo genes. In *Tetrahymena*, a 400 bp sequence interrupts the

Figure 1.1 Organization of rDNA in the eukaryotic nucleus.

A. Structure of the rDNA repeating unit in the nucleus of eukaryotic cells. The mature rRNA sequences are depicted as dark rectangles. The external and internal transcribed spacers regions (ETS, ITS) are labeled and the initial 35-47S transcript is indicated by the thick bar at the top. The open arrows indicate that each rDNA repeating unit is flanked by a non-transcribed spacer region also called the intergenic region.

B. A typical intergenic region in the rDNA repeating unit of eukaryotic cells. Many sequence elements are found within the intergenic region of rDNA. The number and placement of these elements vary according to the organism being studied. The 3' end of the 25S rRNA gene and the 5' end of the 18S rRNA gene are shown as dark boxes and the 5' and 3' transcribed spacers are shown as open rectangles. The possible control elements found within these regions include: 1) termination signals (T), 2) enhancer sequences (E), 3) origin of replication (O) and 4) the two promoter sequences, the upstream control element (U) and core promoter element (C). The transcription initiation site is also indicated (TIS).


A.
Pol I initial transcript 35-47S
5'
3'
5'-ETS
ITS1
ITS2
3'-ETS
17/18S
5.8S
25/28S
Intergenic Region
and upstream
DNA repeats
Intergenic Region
and downstream
DNA repeats


B.
TIS
25S
18S
3'ETS
T
E
O
U
C
5' ETS

coding sequence of the 26S gene approximately 1 kb from the 3' end. This sequence was the first identified group I 'self-splicing' intron (Cech et al., 1981). Approximately 100 other group I introns have been found in the rDNA of lower eukaryotes (reviewed by Johansen et al. 1996).

The 5S pre-rRNA genes are clustered in separate locations from the large pre-rRNA clusters in almost all eukaryotes (Long and Dawid, 1980). The exceptions to this are *Saccharomyces cerevisiae* and a few other fungi which have the 5S located within the NTS of the rDNA repeat (Philippsen et al., 1978) . Like the pol I transcriptional unit that contains the other rRNA genes, the 5S genes are repeated anywhere from 100 to 100,000 copies per haploid genome and may be distributed over several chromosomes (reviewed in Paule and Lofquist, 1996). Each repeated unit in a 5S gene cluster includes a coding sequence and an intergenic spacer and, as with the large rDNA repeat, the location and number of clusters varies according to the species being examined.

C. The mature rRNAs

1. Structure of rRNA

The SSU rRNAs of all organisms fold into complex secondary structures that can contain up to 70 paired elements. Similarly, the 23-28S rRNAs of the large subunit fold into a structure that can contain over 100 paired regions that are arranged in six large domains (Noller, 1984). The rDNA genes for over 1800 SSU (Gutell et al., 1993) and greater than 1000 23-28S rRNAs (Neefs et al., 1993) from organisms representing the three kingdoms have been sequenced. On initial inspection of the primary structure it appears that rRNA is a patchwork of evolutionary conserved sequences interspersed within non-conserved

sequences (Gerbi, 1996). However, universal core secondary structures have been identified for the 16/18S and 23/25S rRNAs (Veldman et al. 1981, Clark et al., 1984). For example, the secondary structure for the *E coli.* 23 S molecule (Noller et al., 1991) was found to be conserved in the yeast 25S rRNA with the yeast molecule having extra blocks of sequence inserted within its core structure relative to the RNA in *E. coli.* Subsequently, it was reported that the 28S molecule of multicellular eukaryotes also has extra blocks inserted into a core structure. The extra blocks occupy the same relative positions in all organisms which have been studied and have been termed expansion segments (Ware et al., 1983) or variable regions (Chan et al., 1983). The increase in size from the *E coli.* 23S rRNA (2904 nucleotides), to the human 28S rRNA (5052 nucleotides) has been attributed to the differences in the number and size of expansion segments (Gerbi, 1996). Expansion segments are also found in the SSU rRNAs and again account for the increase in size from the 16S *E coli.* rRNA to the 18S mammalian SSU rRNA. To date, a total of 12 expansion segments have been found in SSU rRNAs and 41 in the 23-28S rRNAs (Gerbi, 1996).

Unlike the large rRNA molecules, the small rRNAs of the LSU (5.8S and 5S) do not contain expansion segments. Initial secondary structure estimates of the 5.8S rRNA, were performed on this molecule in solution and a generalized 'burp gun' model was accepted (Nazar et al., 1975). However, as stated earlier, within the context of the ribosome, the 5.8S rRNA is not free but is associated with the 25-28S rRNA. When structural estimates are performed for this interaction the structure generated is almost identical to that which occurs when the 5' end of the prokaryotic 23S rRNA folds back onto itself (Nazar, 1980). This has given rise to the hypothesis that during the evolution of eukaryotes the 5' end of the

23S rRNA molecule separated and has persisted as the 5.8S rRNA species.

The 5S rRNA is the most highly conserved rRNA. This small 120 nucleotide molecule folds into four paired helices that form a wishbone-like secondary structure (Fox and Woese, 1975, Garrett et al., 1981). Due to its small size, the higher order structure has been elucidated through mutational analysis followed by chemical modification and enzymatic cleavage reactions (McDougall and Nazar, 1983, Van Ryk and Nazar, 1992). These studies suggest that the tertiary structure of the 5S rRNA from *S. cerevisiae* resembles a lollipop. A lot of attention over the past several years has been given to elucidate the higher order structure of the other rRNAs as well as their context within the ribosome, but to date, very few details have been reported.

2. Functions of rRNA

The first 40 years of ribosomal research focused on determining what r-proteins were important for the ribosomes catalytic activity, and it was thought that the rRNA molecules served simply as a structural framework to which the functionally active proteins bind. However, with the discovery of the catalytic RNA molecules such as group I introns, (Cech et al., 1981) and the catalytic function of the RNA component of RNase P, (a ribonucleoprotein necessary for tRNA maturation in prokaryotes) (Guerrier-Takada et al., 1983) investigators turned their attention to determining the functional roles of the rRNAs.

There is increasing biochemical, genetic and phylogenetic evidence for the functional role of rRNA. This includes all steps of the translation process including: messenger RNA (mRNA) selection, transfer RNA (tRNA) binding, ribosomal subunit association, translocation, proof reading, antibiotic interactions, frame shift suppression,

termination and peptidyl transferase activity (reviewed in Dahlberg, 1989, Noller, 1991 and Green and Noller, 1997). The first and now classic example illustrating the rRNAs role in translation is the base pairing of the Shine Dalgarno sequence in prokaryotic messenger RNAs (mRNA) to a pyrimidine-rich sequence at the 3' end of the 16 S molecule for the recruitment of mRNAs by the ribosome (Shine and Dalgarno, 1974, Hui and deBoer, 1987). Chemical foot printing and crosslinking of tRNAs on rRNA has revealed that there are at least four elements of the 16S rRNA molecule involved at the A site (decoding site) (Moazed and Noller, 1990) and the P site (peptidyl transferase site) is even more disperse with greater than ten 16S rRNA regions making contact with the tRNA molecule (Doring et al., 1994). Originally it was thought that the 16S rRNA did not make contact at the E (exit) site, but Wower et al., (1993), have shown tRNA footprints on the 3' portion of the molecule. U.V. studies have shown that the 16S molecule binds to the mRNA in several places (Rinke-Appel et al., 1994). When the mRNA cross-links are compared with the tRNA footprints, a number of 16S rRNA elements can be placed at the interface of the SSU where translation decoding is hypothesized to occur (Green and Noller, 1997).

One of the most direct lines of evidence that suggests that the largest rRNA species has a functional role was reported by Noller et al., in 1992. In this study, ribosomes from *Thermus aquaticus* were treated with proteinase K and phenol extracted several times such that 95% of protein content was removed. When the remaining LSU particle was assayed for peptidyl transferase activity, activity could still be detected albeit at a reduced level. Further to this, an engineered 1 base substitution (G2251) in the 23S rRNA was shown to decrease peptidyl transferase activity 300 fold (Green et al., 1997). Most recently, it was

demonstrated that *in vitro* prepared 23S rRNA from *E coli.* could promote peptide bond formation in the absence of any proteins (Nitta et al., 1998).Similar to the 16S rRNA studies, tRNA foot printing has revealed that many regions within the 23S rRNA molecule make contact with the tRNA at the A, P and E sites (Moazed and Noller, 1990). Although it is thought that decoding is a function of the 16S rRNA molecule, mutational analysis has revealed that frame shift suppression may be a function attributed to the large rRNA molecule (O'Conner and Dahlberg, 1995). Other functions such as subunit association (Leviev et al., 1995) and termination (Arkov et al., 1998) have been attributed to both rRNA molecules. Although the functions of rRNA have been determined mostly using prokaryotes, the same functions are thought to be performed by the equivalent regions in eukaryotes.

The cytoplasmic ribosomes of eukaryotes contain two additional features, namely the 5.8S rRNA and the variable regions discussed earlier. While our knowledge of the essential features of the core structures in rRNA species has advanced greatly over the last decade, the role of the variable regions remains elusive. However, several recent mutagenesis studies show that certain variable regions in both 17/18S rRNA (Henry et al., 1994, van Nues et al., 1997) and the 25/28S rRNA (Sweeney et al., 1994, Jeeninga et al., 1997) are important for either processing of the primary transcript or in ribosome biogenesis. To date, however, no translational function has been assigned to these areas.

Direct evidence of a functional role for the 5.8S rRNA was first reported in a study that utilized antisense oligonucleotides complementary to regions of the *Schizosaccharomyces pombe* 5.8S rRNA that were thought to be exposed on the surface of the ribosome (Walker et al., 1990). The results of these studies demonstrated that the

antisense oligomers could specifically inhibit protein synthesis *in vitro*. In a subsequent study, a five base insertion was engineered to disrupt a universally conserved four base sequence (Abou Elela et al., 1994). This report showed that protein synthesis was inhibited both *in vitro* and *in vivo* to the same extent. It was also shown that there was also an increase in polyribosomal profiles and an increased amount of tRNA in these profiles which suggested that 5.8S rRNA plays a role in protein elongation. This work was supported further when the mutant ribosomes were tested with specific inhibitors of protein synthesis and it was determined that the translocation step was impaired (Abou Elela and Nazar, 1997). To date the similar region in the 23S rRNA molecule has not been examined to determine if the same effects are seen.

The 5S rRNA molecule which is the most highly conserved rRNA is ubiquitous to nearly all ribosomes and has been implicated as having a role in virtually every important ribosomal function (for review see Nazar, 1982). However, contrary evidence has been presented from a number of studies which question each of these roles (Garrett 1979, Lo and Nazar, 1982, Goringer et al., 1984, Zagorska et al., 1984, Christiansen et al., 1985, Egebjerg et al., 1989 and Van Ryk et al., 1990). Yet, it is known that, in *S. cerevisiae,* if mutations are generated such that the 5S molecule is not incorporated into the LSU, the subunit becomes unstable and quickly degrades (Deshmukh et al., 1993). Two recent studies show promise in elucidating a translational function for 5S rRNA (Dokudovskaya et al., 1996, Sergiev et al., 1998) . In both studies, 5S rRNA was transcribed *in vitro* in the presence of nucleotide analogues which when activated by ultraviolet light, were cross-linked to neighboring RNA molecules. These 5S rRNA analogues were reconstituted into 50S or 70S ribosomes, then

irradiated. In both studies a single base (U-89) was cross-linked to at least two sites on the 26 rRNA. In the later study, the base contacts the region of 26S rRNA thought to be the GTPase center (necessary for elongation). Still, a distinct function for this rRNA species remains to be defined.

D. Transcription of rRNA

1. The nucleolus

Collectively the most active transcriptional unit in the cell is that which encodes the pre-rRNA genes. In growing eukaryotic cells, transcripts from the rRNA genes can account for up to 80% of all the RNA synthesized at any moment. The interphase nucleus contains varying numbers of nucleoli which are membraneless substructures that serve as the sites of rDNA transcription, rRNA processing and ribosomal subunit biogenesis (reviewed by Reeder, 1990, Sollner-Webb and Mougey, 1991, Hannan et al., 1998). The ribosomal genes are the central elements of the nucleolus and recent studies suggest that the nucleolus is simply the assembly of molecules bound to the rRNA genes, their transcripts and associated proteins (Nierras et al., 1997). Surrounding the rRNA genes is a fine network of scaffold filaments that are distinguishable from the nuclear matrix. This scaffolding is hypothesized to provide some structural support or organization for the arrangement of transcriptionally active rDNA, or to have an involvement in the assembly or transport of the ribosomal subunits (Reeder, 1990).

Nucleoli consist of three parts which have been named according to their appearance in electron microscopy ; Fibrillar Centers (FC), Dense Fibrillar Component (DFC) and Granular Component (GC) (reviewed in Larson et al., 1991, Schwarzacher and Wachtler,

1993). It has been thought that transcription occurs in the FC, rRNA processing in the DFC and subunit assembly in the GC based upon localization studies of transcription factors and components necessary for processing (Roussel et al., 1993, Matera et al., 1994). More recent studies that utilized *in situ* hybridization and confocal microscopy to detect pre-rRNA in mouse cells suggest that : 1) the FC is devoid of rDNA except at the periphery, 2) the DFC is not a continuous compartment but may actually have three subregions where transcription and some processing events occur and 3) further processing events and subunit maturation occur in the GC (Lazdins et al., 1997). These regions in lower eukaryotes are not as clearly defined, possibly due to resolution difficulties given their smaller size. Nevertheless, it is felt that the same substructures exist (Leger-Silvestre et al., 1997).

2. The rRNA gene promoter

Transcription of rDNA by polymerase I requires at least two sequence elements that are recognized by numerous trans-acting factors (reviewed by : Paule, 1993, Moss and Stefanovsky, 1995, Paule and Lofquist, 1996, Hannan et al., 1998). Despite the lack of recognizable sequence homologies between eukaryotic rRNA promoters (except in vary closely related species), the overall architecture of all promoters is nearly identical. The essential elements have been studied by : deletion analysis (Moss, 1982, Grummt, 1982, Hayward and Glover, 1988), linker scanning (Musters et al., 1989, Choe et al., 1992) and point mutation (Jones et al., 1988, Firek et al., 1990, Read et al., 1992) in mammals, amphibia, diptera, protozoa, and yeast. These studies have defined the two essential sequence elements as the core promoter element (CPE) and the upstream control element (UCE). The core promoter element is found in all eukaryotic organisms and, in general,

extends from approximately 10 bp downstream to 40 bp upstream of the transcription initiation site (tis). It can be divided into two subregions, an initiator segment which includes the tis and an upstream element that binds one or more trans-acting factors. The UCE is found farther upstream and generally extends to approximately nucleotide -150. Studies performed on rat, *Xenopus* and *S. cerevisiae* suggest that the spacing between the UCE and core elements is crucial (Windle and Sollner-Webb, 1986, Xie et al., 1992, Choe et al., 1992). Mutations which introduced or deleted five bp severely impaired transcription whereas deletions or insertions that were a multiple of ten (ie. a helical turn), had less dramatic effects. This has lead to the hypothesis that the two elements have to be correctly aligned for proper function (Choe et al., 1992).

Other sequences involved in promotion of rDNA transcription include: promoter proximal terminator elements and enhancers. As originally observed in *Xenopus* and later in mouse and *S. cerevisiae*, rDNA promoters of some organisms are closely preceded by a transcription terminator element (Moss, 1983, Grummt et al., 1986a). It is felt that these elements function as a safe guard, in that they shield downstream promoters from being occluded by upstream transcription complexes that are not properly terminated (Bateman and Paule, 1988).

Enhancer sequences also have been determined for most of the organisms which have been studied. Close inspection of intergenic regions of higher eukaryotes revealed repeated elements of varying size and sequence depending on the organism. These repeat units were shown to be enhancer sequences for pol I transcription (Labhart and Reeder, 1984, Pikaard et al., 1990, Grimaldi et al., 1989). The sequences are termed enhancers

because they function similarly to pol II enhancer sequences; they are orientation independent and can function at a distances (Labhart and Reeder, 1985). The yeasts, *S. cerevisiae* and *S. pombe,* differ from the higher eukaryotes in the fact that within their intergenic regions there exists only one enhancer element (Elion and Warner, 1986, Zhao et al., 1997). The reason behind these differences remains unclear and in all cases the mechanism by which these enhancer elements function remains to be elucidated.

3. Proteins that mediate rDNA transcription

There are three main protein complexes that mediate pol I transcription which include: the pol I complex itself, an upstream binding factor (UBF) and a TATA binding complex (TBP_I). RNA polymerase I is a large complex enzyme with an approximate molecular weight of 500,000-600,000 Daltons (Hannan et al., 1998). Two recent studies report that mammalian Pol I is composed of at least 12 subunits and three associated factors whereas the yeast equivalent consists of at least 14 subunits of which all have been cloned (Hannan et al., 1998b, Hanada et al., 1996, Gadal et al., 1997). To date, the functions of these subunits is unclear, however, it has been shown that most of the subunits are essential for viability in *S. cerevisiae* (Sentenac et al., 1992).

There is evidence that several rDNA transcription factors may interact with the core promoter element, but, TBP_I (also called: SL1, TIF-IB, CF, TFID, factor D or Rib 1) is considered to be the primary interacting complex (Hannan et al., 1998). TBP_I consists of the TATA-binding protein (TBP) and at least three RNA pol I specific TBP associated factors (Comai et al., 1994). Although TBP_I is the primary interacting factor of the core promoter, TBP_I by itself cannot form a stable DNA-protein complex. However, in the presence of UBF

bound to the UCE, TBP_I binds to the core promoter and directs the initiation of transcription. (Keys et al., 1996).

UBF is a phosphoprotein that contains four to six high mobility group boxes and binds as a dimer to the CPE, UPE and enhancer elements in the intergenic region of rDNA (Jantzen et al., 1990, Pikaard et al., 1990, Xie et al., 1992). More recently, it has become evident that UBF binds to the UCE of the rDNA and by inducing folding and bending, shortens the DNA by approximately 190 bp. This generates a disk-like UBF-rDNA complex that has been termed the "enhancersome" (Bazett-Jones et al., 1994). The bending of the rDNA promoter then allows TBP_I to bind the CPE generating the pre-initiation complex which then recruits pol I and transcription begins. Other trans-acting factors that may also play a role in transcription include Ku/E1BF, CPBF (may bind the core promoter), topoisomerases, and p16 (a protein that may stimulate rDNA transcription) (reviewed in Hannan et al., 1998).

4. Regulation of rDNA transcription

Although ribosome synthesis can theoretically be regulated at numerous steps, most studies show that regulation occurs by controlling pol I transcription of the rDNA. Transcription of the rDNA can be regulated in several ways which include: chromatin accessability, alteration in the amounts or activity of pol I or alteration in the amounts or activity of other trans-acting factors (Hannan et al., 1998). Chromatin may regulate gene activity by limiting access of transcription factors to the DNA, however, UBF can bind either nucleosomal or naked DNA equally and the role of chromatin structure in rDNA transcription remains unclear (Moss and Stefanovsky, 1995). Studies of the pol I subunits,

the pol I associated factors and the trans-acting factors (other than TBP_I and UBF) have shown correlations of pol I activity and their respective levels. But to date, these studies have in most cases been performed *in vitro* and the functions of these proteins in regulation of rDNA transcription are yet to be determined.

One might predict that TBP_I is a potential target for transcriptional regulation of rDNA due to its function, yet there has only been one report of an altered level or activity of this necessary complex (Zhai et al., 1997). However, many studies have linked altered levels or activity of UBF with ribosome biogenesis. For example, during the differentiation of mouse myoblasts into myotubules there is a correlated decrease in the amount of UBF in these cells (Larson et al., 1993). Also, serum starvation of mouse 3T3 cells was shown to be accompanied by a decrease in UBF concentrations and, upon serum restoration, UBF levels increase (Glibetic et al., 1995). In another study, when cardiomyocytes were induced to proliferate there was a concomitant increase in the UBF levels which was correlated with increased levels of ribosome biogenesis (Hannan et al., 1995).

The phosphorylation state of UBF can regulate its activity with the hyperphosphorylated form being the most active (Voit et al., 1992). Stimulation of cardiomyocytes to proliferate was shown to be accompanied by an increase in the phosphorylation of UBF and in other studies serum depravation of cell culture lines show a conversion of UBF to the hypophosphorylated form (O'Mahony et al., 1992, Luyken et al., 1996). Also, UBF can be sequestered in an inactive form by the cell cycle control and tumor suppressor gene, Rb^{110}. It was reported that when U937 cells were induced to differentiate, Rb^{110} accumulated in the nucleolus, and rDNA transcription decreased in the absence of

UBF concentration decreases (Cavanaugh et al., 1995). Also, in this study when antibodies against Rb^{110} were used, UBF was co-immunoprecipitated. This suggests that UBF activity is regulated during the cell cycle. It would, therefore, appear that the cell has developed several mechanisms for regulating the activity of UBF and, thereby, regulating rDNA transcription.

5. Termination of rRNA transcription

It is well established that if pol I transcribing an upstream rDNA repeat unit continues over the promoter of the next repeat downstream, the downstream promoter is occluded and shutoff (McStay and Reeder, 1990). Every pol I transcription unit (with the exception of *Drosophilas*) has within the intergenic region a specific terminator sequence which has been hypothesized to prevent this occlusion from occurring (Reeder and Lang, 1994). Termination of pol I appears to be a multi step process involving pausing of the polymerase followed by release of the new transcript and the polymerase from the template and finally 3' end processing of the newly released pre-rRNA. In mouse, human, *Xenopus laevis*, *S. cerevisiae* and *S. pombe* the sequence elements that are functionally important for transcription termination have been identified and in most organisms these termination sequences are tandemly repeated (Grummt et al., 1985, Bartsch et al., 1988, Labhart and Reeder, 1986, Lang and Reeder, 1993, Melekhovets et al., 1994). All characterized pol I terminator elements, while different in sequence, are similar in that they are orientation and trans-acting factor dependent. Further similarities lie in the fact that they all direct rRNA 3' end formation 11- 21 nucleotides upstream from their 5' end (Reeder and Lang, 1994).

The cDNAs that code for the proteins that mediate termination have been cloned for

mouse (mTTF), *S. cerevisiae* (Reb 1p) and *S. pombe* (SpReb 1p) (Evers and Grummt., 1995, Morrow et al., 1993, Zhao et al., 1997). Each of these proteins has the ability to bind the termination element and stop pol I elongation *in vitro* (Kuhn et al., 1990, Lang et al., 1994, Zhao et al., 1997). Initially there appeared to be some discrepancy in terms of how this is accomplished mechanistically. For example, it was well documented in *S. cerevisiae*, that the Reb 1p protein binds to its cognate termination element and stops pol I. However, complete termination (pol I pausing and release of the new transcript) was shown to require the presence of 5' flanking sequences which included a stretch of thymidine residues (Jeong et al., 1995). This was in apparent contrast to the mouse system where a repositioned terminator element could direct proper 3' end formation without its natural flanking sequences (Kuhn et al., 1988). However, recent studies in these two systems suggest that the mechanism has been evolutionarily conserved (Mason et al., 1997). The emerging picture indicates that the terminator proteins bind to their cognate termination elements to pause pol I and then, in concert with a release factor (Mason et al., 1997a), and 5' flanking sequences (which include several thymidine residues) are able to release the nascent transcript (Mason et al., 1997). The caveat with this work is that it has all been performed *in vitro* and it remains to be established if similar results will be seen *in vivo*.

The only *in vivo* studies of termination to date have been performed with *S. pombe* (Melekhovets et al., 1997). In this work it was reported that when the first termination element (where 90% of termination occurs) was repositioned upstream of its natural site, termination occurred efficiently suggesting that the flanking sequences are not important. However, further studies of the element in the natural sequence context have shown that

when 5' flanking sequences are disrupted termination at this site is abolished (Shwed, 1998).

E. Processing of rRNA

1. Pathways and cleavage sites

The sequence of processing events that leads to the formation of mature rRNA species has been studied extensively for many years and, in general, occurs in a polar fashion from the 5' to 3' end of the primary transcript. However, the order and intermediates generated during rRNA processing can vary between species and even within a given cell when growth conditions are altered. For example, in rat liver (Dudov and Dabeva, 1983), *Xenopus* oocytes (Gerbi et al., 1990, Peculis and Steitz, 1993), and mammalian cells (Bowman et al., 1981, Hadjiolova et al., 1993) multiple processing pathways have been reported. The relationship of these altered pathways to ribosome biogenesis is still not understood. Figure 1.2 is a schematic representation of the rRNA transcriptional units, the main cleavage sites and the processing pathways that have been reported for *S. cerevisiae* (1.2A)van Nues and Craig, 1994). It is important to note that in many cases it has not been established whether the cleavage sites depicted are due directly to an endonucleolytic cleavage, or whether the intermediates result from the trimming action of an exonuclease that was initiated from some other endonuclease site. Also, only those intermediates whose steady state levels are sufficiently abundant to be detected can be distinguished as an intermediate. As a result, some true intermediates that are quickly processed may remain unrecognized. In general, the inter-transcribed spacer cleavages are endonucleolytic in nature and the mature ends of the rRNAs are generated by exonucleolytic digestion.

Removal of the 5' ETS from the pre-RNA transcript is by far the most studied

processing step. Initially, it was thought that the 5'ETS was removed in its entirety by an endonucleolytic cleavage reaction at the A1 site, generating the mature 5' end of the 18S rRNA. However, in 1981, Miller and Sollner-Webb reported that the 5' terminal 650 nucleotides of the mouse primary transcript were removed very rapidly. This site was termed the "primary processing site" and the analogous sites in humans (Kass et al., 1987), frogs (Mougey et al., 1993a), yeast (A0) (Hughes and Ares, 1991) and *S. pombe* (A0) (Good et al., 1997) have been identified. Although other cleavage sites in the 5' ETS of mouse generally are not included in diagrammatic representations, other processing intermediates have been reported (Mishima et al., 1985, Raziuddin et al., 1989). More recently, Good and co-workers utilized primer extension analysis to show that at least six transient termini are generated during removal of this spacer in *S. pombe*. The mechanism by which the mature 5' terminus of the 17/18S rRNA is formed is unclear, however Venema and co-workers (1995) reported that structural elements within the mature rRNA are important. In any event, processing of this spacer appears to be far more complex than previously was believed.

Initial studies of the 3' ETS failed to detect processing at the 3' end of the pre-rRNA and it was assumed that the polymerase terminated at the mature end of the 25/28S rRNA or at the very most was extended beyond this site by only a few nucleotides (reviewed by Platt, 1986). More recently, many studies using sensitive nuclease protection assays and nuclear run-off experiments have shown that in a variety of organisms, transcription extends into the intergenic spacer and rapid processing occurs to form the mature 3' termini of the

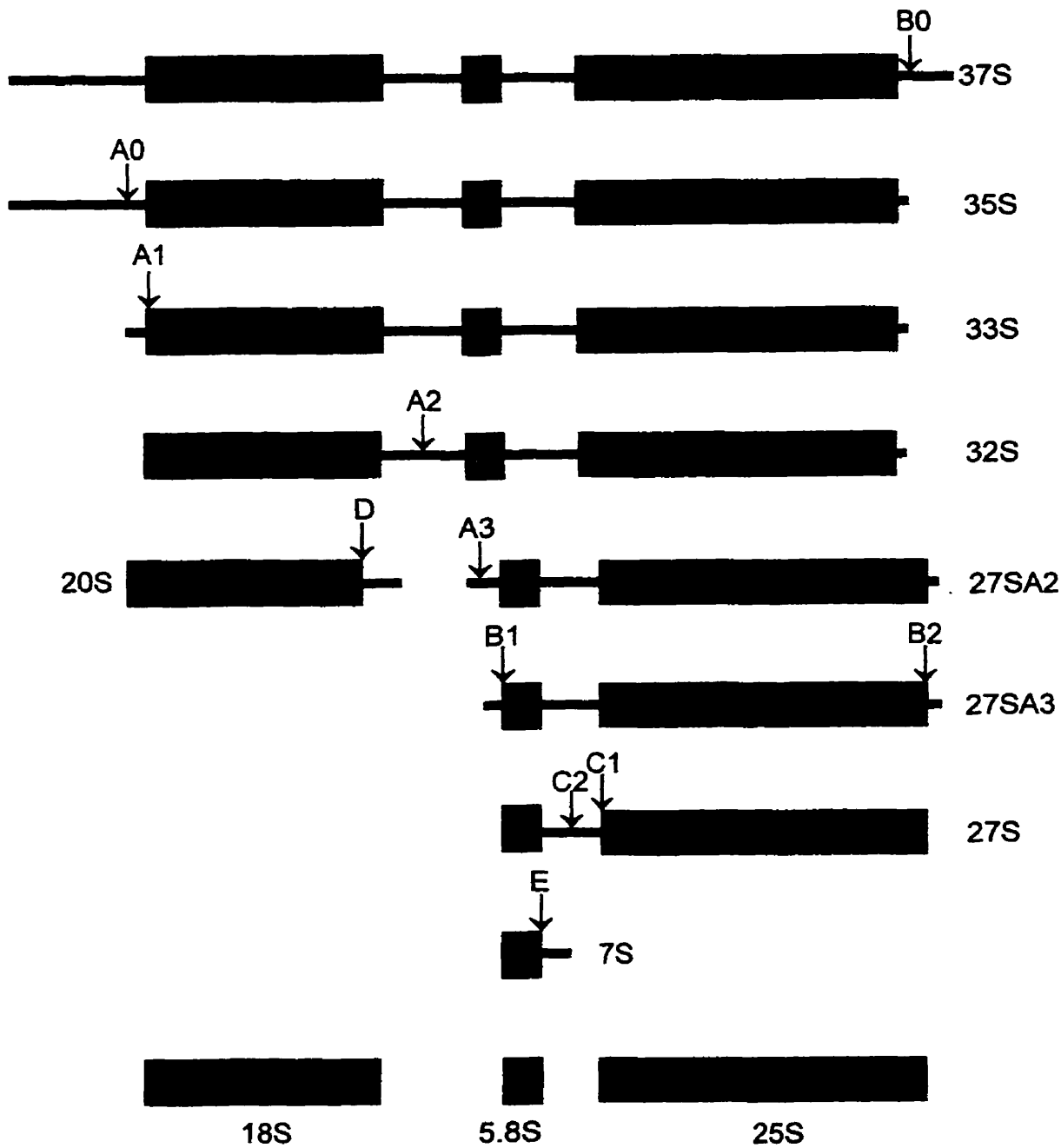
Figure 1.2 rRNA maturation pathways in commonly studied organisms.

A. The rRNA maturation pathway of *S. cerevisiae* (van Nues et al., 1995). The processing steps are indicated by solid arrows and are labeled according to Veldman et al., 1981.

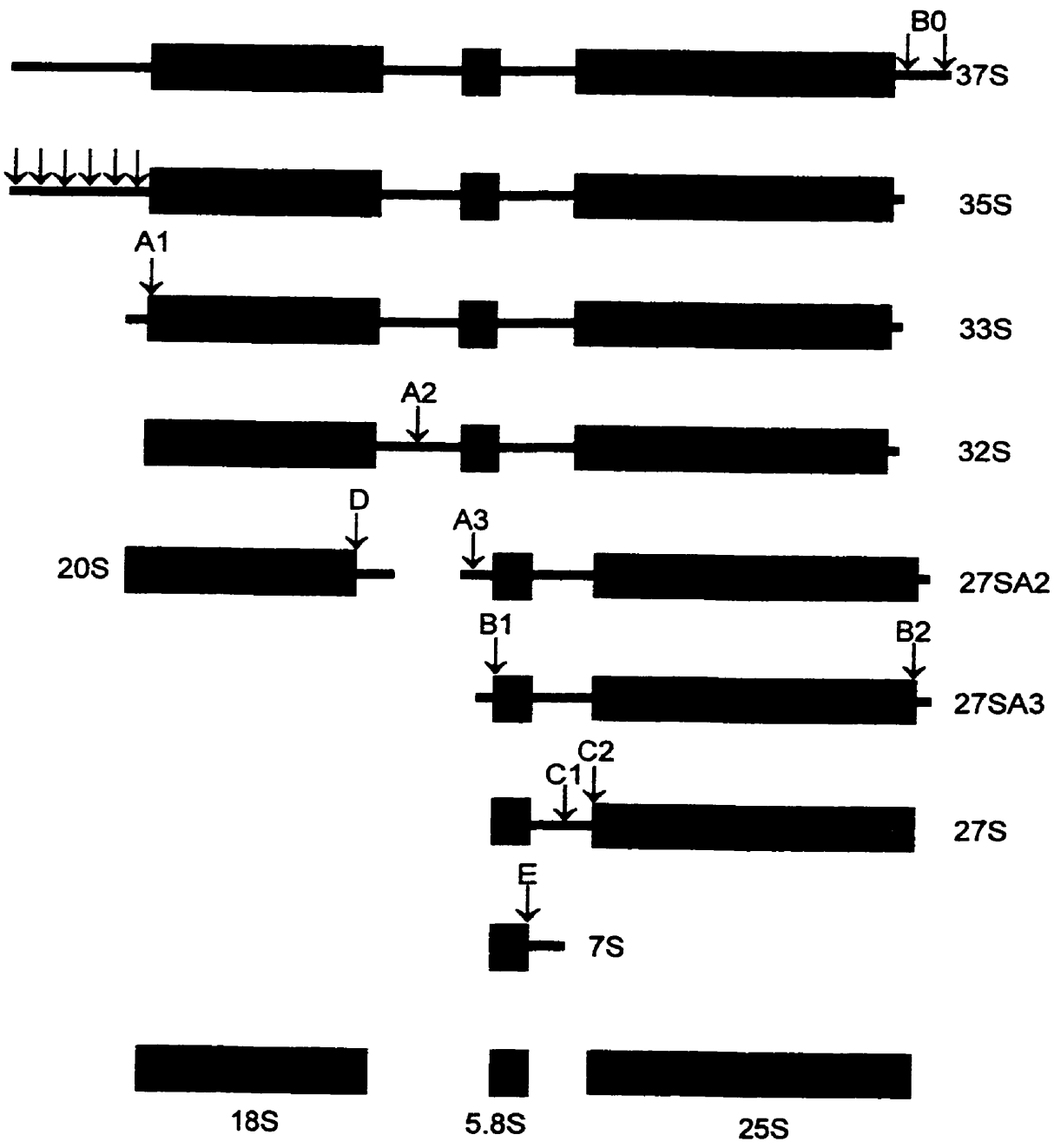
B. Steps in the processing pathway of *S. pombe* (Good et al., 1997).The processing steps are indicated by solid arrows and are labeled according to Veldman et al., 1981.

C. The rRNA processing pathway of a typical higher eukaryote (mouse) (Eichler and Craig, 1994). The processing steps are indicated by solid arrows and are labeled by number according to the order they are hypothesized to occur.

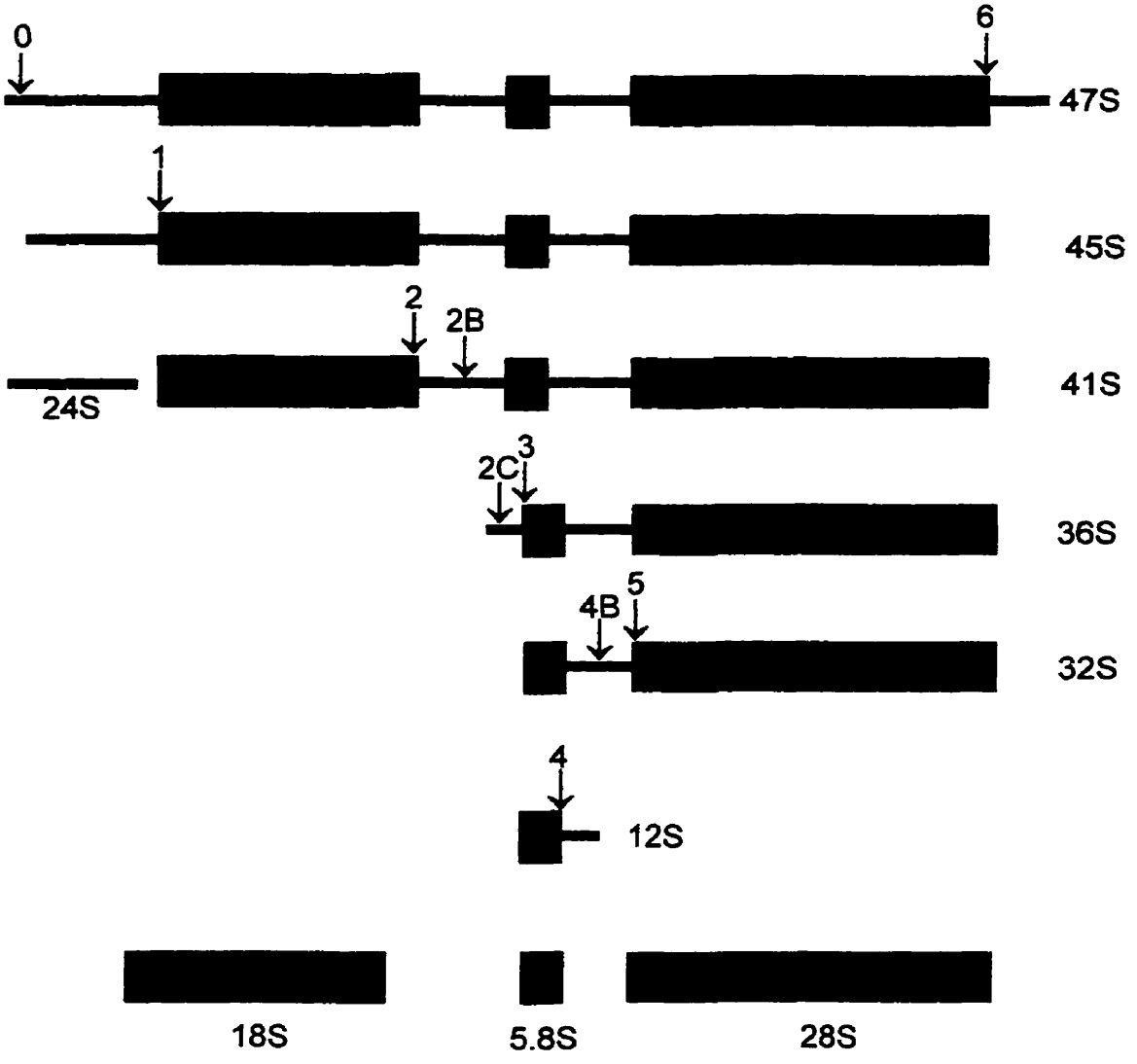
A.


B0
37S
A0
35S
A1
33S
A2
32S
D
A3
20S
27SA2
B1
B2
27SA3
C2
C1
27S
E
7S
18S
5.8S
25S

B.


B0
37S
35S
A1
33S
A2
32S
D
A3
20S
27SA2
B1
B2
27SA3
C2
C1
27S
E
7S
18S
5.8S
25S

C.



25/28S rRNA. For example, in *S. cerevisiae* the 3' ETS is approximately 210 nucleotides long and there are processing events that generate three intermediate termini (Kempers-Veenstra et al., 1986, van der Sande et al., 1989). Similarly three transient termini can be detected for the removal of the 267 nucleotide 3' ETS in *S. pombe* (Melekhovets, 1994). Removal of this spacer in the frog or the mouse is not well understood and only one intermediate has been detected in either species (Kuhn et al., 1988, Labhart and Reeder, 1990).

Removal of both internal transcribed spacers appears to be the most evolutionarily conserved in terms of the number of cleavage reactions that are required. These events are best characterized in *S. cerevisiae* and serve as a model that is generally accepted (Lygerou et al., 1994). After the 5' ETS is removed and most of the 3' ETS, cleavage at A2 within ITS1 generates the 20S and 27SA precursors (See Fig. 2A). Further maturation of the 20S precursor to yield the mature 17/18S rRNA is thought to be by endonucleolytic cleavage and has been reported to occur in the cytoplasm (Stevens et al., 1991). The 27SA precursor is shortened initially by an additional cleavage in ITS1 at the A3 site. Recent studies have shown that the cleavages at A2 and A3 are linked and both are necessary for efficient processing (Allmang et al., 1996a). The rest of the ITS1 sequences are removed by exonuclease trimming to generate the heterogeneous mature 5' end of the 5.8S rRNA (site B1). The mechanism that stops the exonuclease action remains unknown but it has been hypothesized that r-proteins may bind to the mature 5.8S rRNA sequences and block further action by the putative exonuclease (Henry et al., 1994, Lee et al., 1995). An alternative route for ITS1 processing also exists which produces a 5.8S rRNA with seven to eight extra

nucleotides on the 5' end (Lygerou et al., 1994, Allmang et al., 1996b). In this case, when cleavage at A3 is inhibited, the long form of the 5.8S rRNA is produced directly by endonucleolytic cleavage. This second mechanism is thought to function normally, as up to ten percent of 5.8S rRNA isolated from *S. cerevisiae* cells is of the long form (Rubin, 1973, Lygerou et al., 1994).

Similar to ITS1 processing, ITS2 undergoes a cleavage within the spacer (site C1). This event releases the 5.8S rRNA with a 3' extension, the 7S intermediate, which is relatively long lived before cleavage at site E (Peculis and Steitz, 1993). On the other hand, cleavage at C2 (which forms the mature 5' terminus of 25S rRNA) occurs very rapidly after or concomitantly with C1, as no intermediates with extensions on the 5' end of 25S rRNA have been detected in *S. cerevisiae* (Veldman et al., 1981, Reeder, 1990). However, recent studies in *S. pombe* show that an intermediate with the 5' extensions can be detected. Also, similar to the 5.8S rRNA, the 5' terminus of the 25S rRNA is heterogeneous with stable extensions up to eight nucleotides long (Good et al., 1997b). This suggests that similar mechanisms may function for the removal of ITS1 and ITS2.

2. Trans-Acting factors

Much of our understanding of the underlying mechanism of pre-rRNA processing has come from the identification and analysis of trans-acting factors. A variety of experimental approaches have been applied including: 1) the analysis of yeast mutants defective in rRNA processing 2) *in vitro* analysis using synthetic precursor molecules and cellular extracts and 3) the inactivation of small nucleolar RNA species (snoRNP) by injection of antisense oligonucleotide into *Xenopus* oocytes. These studies have lead to the

identification of three general classes of trans-acting factors: snoRNPs, catalytic proteins and non-catalytic proteins. As is the case with all aspects of ribosome biogenesis, the number and function of trans-acting factors is best understood for *S.cerevisiae* due to the ease with which genetic manipulations can be performed.

a. SnoRNAs

A rapidly increasing number of snoRNAs are being characterized and are shown to be required for pre-rRNA processing (reviewed in LaFontaine and Tollervey, 1995, Maxwell and Fournier, 1995, Tollervey, 1996). The earliest and best understood processing events of pre-RNA are in the removal of the 5' ETS and subsequent maturation of the 18S rRNA. Both in mammalian cells and in *S.cerevisiae* it was known for some time that U3 RNA could be cofractionated with pre-RNA in deproteinized extracts, consistent with the fact that it was hydrogen bonded to the pre-RNA (Prestayko et al., 1970, Tollervey, 1987). *In vivo* psoralen cross-linking studies also have been utilized to show that the U3 snoRNA is hydrogen bonded to the pre-RNA several hundred nucleotides from the primary cleavage site in human (Maser and Calvet, 1989), rat (Stroke and Weiner, 1989) and *S. cerevisiae* (Beltrame and Tollervey, 1992).

Initially, studies showed that the U3 snoRNP was essential for processing of the early cleavage site in mammalian pre-RNA *in vitro*. Depletion of U3 snoRNP by immunoprecipitation or oligonucleotide directed cleavage of the snoRNA interfered with the cleavage of the 5' ETS (Kass et al., 1987, Kass et al., 1990). Subsequently, RNase H oligonucleotide directed cleavage of the U3 snoRNA also inhibited *in vitro* 5' ETS removal in *Xenopus* (Mougey et al., 1993a). The U3 snoRNP is essential for viability in

S. cerevisiae (Hughes et al., 1987). Interference with its function results in a block in the formation of the mature 18S rRNA due to a loss of cleavages at A0, A1 and A2. A U3 binding site has been identified at a position 230 nucleotides upstream of the A1 site. Ten nucleotides of the pre-RNA at this site have the potential to form ten perfect base pairs to a portion of the U3 snoRNA. Deletion or substitution of these complementary nucleotides abolishes cleavage of the pre-rRNA and mimics the earlier U3 depletion studies (Beltrame et al., 1994). Expression of mutant U3 snoRNAs which restore base pairing in this area restores processing at all of the sites (Beltrame and Tollervey, 1995). Therefore it would appear that the base pairing between the U3 snoRNA and the pre-rRNA is a prerequisite to processing, yet, the exact function of this trans-acting factor remains to be elucidated. Recently, it has been reported that the U3 snoRNP actually may play a role in the formation of the universally conserved loop/stem pseudoknot structure that forms three bases in from the 5' end of the 18S rRNA (Hughes, 1996).

. Similar to the U3 snoRNA, U14 snoRNA, also has been cross linked to pre-rRNA; in this case it hybridizes with sequences of the mature 18S rRNA (Trinh-Rohlik and Maxwell, 1988, Li et al., 1990). The U14 RNA molecule is comprised of three domains termed A, B and Y (Morrissey and Tollervey, 1997). Domain B, conforms to the consensus for methylation guide snoRNAs (discussed later) and is predicted to perform this function (Kiss-Laszlo et al., 1996). However, unlike the methylation guide snoRNAs, U14 is essential for processing and depletion or mutation of this RNA inhibits processing at A0, A1 and A2 (Hughes and Ares, 1991). In fact, domain B is dispensable for processing, but domain A is necessary. The essential contact site of domain A with the pre-rRNA remains unknown,

although recently several contact points have been reported (Morrissey and Tollervey, 1997). Several other snoRNAs also have been reported to play a role in the removal of the 5' ETS. In yeast, genetic depletion of snR10 slows down the processing of the pre-rRNA and a buildup of full length precursor molecules is seen (Tollervey, 1987). Also, recently in *Xenopus*, E1, E3 and U22 snoRNPs, were proven to be essential for cleavages in the 5' ETS (Enright et al., 1996, Mishra and Eliceiri, 1997). In all cases none of these have been reported to effect the maturation of the large subunit rRNAs.

When visualized by the Miller spreading technique, transcribing ribosomal chromatin is known to form a Christmas tree-like structure with the rDNA as the trunk and rRNA branches of increasing length that are decorated with "terminal balls" (Miller and Beatty, 1969). These terminal balls have been seen in every eukaryotic organism studied to date and are known to be comprised of large ribonucleoprotein complexes (reviewed in Mougey et al., 1993b). The fact that all of the above trans-acting factors are required for the same events (5' ETS processing) combined with the fact that complex RNPs are seen when electron microscopy studies are performed, has lead to the hypothesis that all these factors work in concert for 18S rRNA maturation and collectively have been termed the "processome" (Mougey et al, 1993b).

In contrast to 5' ETS processing, processing of the other spacers does not appear to be dependent on many snoRNP complexes. The yeast 5.8S rRNA is heterogeneous at the 5' end as a result of two alternative processing pathways described earlier. The major species are cleaved endonucleolytically at site A3 by the snoRNA RNase MRP (Henry et al., 1994, Lygerou et al., 1994). Cleavage at A3 is inhibited by either mutation in the RNA

molecule, or in the protein component Pop1p (Shuai and Warner, 1991, Lygerou et al., 1994) and more recently the catalytic function of this snoRNP was shown to be attributed to the RNA molecule (Lygerou et al., 1996). RNase MRP is the only endonuclease activity in pre-rRNA processing that has been assayed directly *in vivo*.

Work carried out with *Xenopus* oocytes shows that the maturation of 28S rRNA also is dependent on a snoRNP. Injection of antisense oligonucleotides directed against specific regions of the U8 snoRNA inhibited processing at the internal cleavage site within ITS2, and at both mature termini of the 28S rRNA (Peculis and Steitz, 1993). This is the only organism in which the U8 snoRNP has been identified in and it is unclear why this organism appears to have evolved a separate mechanism for processing in this area.

b. Catalytic Proteins

i). Endonucleases

The dependence on the snoRNPs for cleavages at A0, A1 and A2 has been clear for several years, however the enzymes that produce these endonucleolytic cleavage events remain elusive. A crude protein extract cleaves the vertebrate 5 ' ETS in a U3 dependent fashion *in vitro* (Kass et al., 1990, Mougey et al., 1993) but the enzymes responsible have yet to be isolated. Also, nucleolar endonucleases that cleave vertebrate 5' ETS transcripts *in vitro* have been isolated but their roles in pre-RNA processing are still not determined (Lasater and Eichler, 1984, Shummard and Eichler, 1988). Recently, the *S. cerevisiae* RNase III (a double strand specific prokaryotic rRNA processing enzyme) homologue, RNT 1, has been tested for its ability to perform these cleavages (Abou Elela et al., 1996). In this study, disruption of this gene showed that it was essential for growth, and a strain carrying a

temperature sensitive allele was unable to process pre-rRNA when shifted to the non-permissive temperature. It was also reported that this enzyme was able to faithfully cleave an *in vitro* derived substrate at the A0 site. However, this cleavage was U3 independent and therefore, while this enzyme clearly plays a role in pre-RNA processing, the cleavage at A0 remains suspect. Unfortunately an endonuclease that generates the mature 5' end of the 18S rRNA has not been reported.

Abou Elela et al. also reported that the endonuclease product of the RNT 1 gene is responsible for the efficient cleavage of the 3' ETS in yeast. The 210 nucleotide spacer is first endonucleolytically cleaved between +15 and +50 and this is followed by exonucleolytic degradation to +7 and finally an endonucleolytic cleavage at the mature 3' termini of the 25S rRNA (Kempers-Veenstra et al., 1986). Abou Elela and co-workers observed that a reproducible cleavage at + 21 was generated when purified rnt-1p was used to cleave an artificial 3' ETS substrate. The RNase III homologue has also been cloned from *S. pombe* (PAC 1) (Xu et al., 1990, Iino et al., 1991). *In vitro* studies of the pac 1 endonuclease have shown that it cleaves two of the three 3'ETS processing sites defined *in vivo* by Melekhovets et al. for *S. pombe* (Rotondo et al., 1997). The enzyme responsible for the formation of the 3' end of the 25S rRNA remains elusive. Initially it was thought that the product of the RNA82 gene in yeast was responsible for this cleavage event due to a lack of 3' end formation in cells harboring mutants of this gene (Piper et al., 1983). Since this initial observation, nothing has been reported on the cloning of this gene or implicating its role directly in this cleavage event. Although several reports have emerged that show promise in identifying the endonucleases responsible for pre-rRNA processing, the only one

characterized to date is the snoRNP MRP described in the above section.

ii). Exonucleases

It has been shown that the gene products that are encoded by the XRN 1 and RAT 1 genes from yeast have 5'-3' exonuclease activities *in vitro* (Larimer et al., 1992, Amberg et al.,1992). Endonucleolytic cleavage of the 5' ETS releases a fragment that spans from A0 to A1. This fragment is degraded by the Rat 1p and Xrn 1p exonucleases as the fragment is detected in greater quantities in either single mutant strain, or increasingly in a double mutant strain (Petfalski et al., 1998). Similarly degradation of the A2-A3 fragment generated during ITS1 processing is also increased in these mutant strains. These exonucleases also appear to be responsible for generation of the 5' end of the 5.8S rRNA as increased levels of precursor molecules that extend out to the A3 site are detected in these mutant strains (Henry et al., 1994). An exonuclease also has been isolated that is responsible for 3' end formation of the 5.8S rRNA. In temperature sensitive mutants of the RRP 4 gene the 7S precursor accumulates and *in vitro* the gene product was shown to possess 3' 5' exonuclease activities (Mitchell et al., 1996). Subsequently it was shown that the trimming of the 7S precursor to yield the mature 5.8S rRNA is performed by a complex of five exonucleases termed the "exosome" of which the RRP 4 gene product is a part of (Mitchell et al., 1997).

iii). Helicases

The RNA sequences of the primary transcript are thought to be highly base paired and processing is dependent on RNA-RNA interactions involving the snoRNA molecules. Therefore not surprisingly, proteins that belong to the DEAD- box family of ATP-dependent helicases have been reported to be involved in rRNA processing in *S. cerevisiae.*

Characterization of *S. cerevisiae* strains that were impaired in ribosome biogenesis revealed at least two putative helicases, DRS 1 and SPB 4 required for 25S rRNA maturation (Sachs and Davis, 1990, Ripmaster et al., 1992). Recently more attention has been given to this family of proteins. Three members were shown to be involved in 18S rRNA maturation [Rok 1p (Tollervey, 1997), Rrp 3p (O'Day et al., 1996), Fal 1p (Kressler et al., 1997)] and Dbp 7p is required for maturation of the 27S precursor (Daugeron and Linder, 1998). In addition, the helicase encoded by the DOB 1 gene affects maturation of the rRNAs of both subunits (de la Cruz et al., 1998). As focus has just recently been placed on this family of proteins, further studies will be required to document the actual role of these proteins in rRNA processing.

c. Non- catalytic proteins

i). Ribosomal proteins

Processing of pre-RNA occurs at the level of RNP particles, hence r-proteins are obvious candidates as trans-acting factors. The first report of this nature came in 1987 when it was shown that disruption of the non-essential S37 protein (*S. cerevisiae*) affected the efficient formation of 40S subunits by retarding the conversion of the 20S precursor into the mature 18S rRNA (Ozkaynak et al., 1987, Finley et al., 1989). Recently, the yeast homologue to the vertebrate S27 r-protein has been isolated and was shown to be essential for 5' ETS removal and subsequent 18S rRNA maturation (Baudin-Baillieu et al., 1997). Three large subunit r-proteins also were shown to have an effect on pre-rRNA processing. Perturbation of the nuclear import of L29 causes a defect in the processing of the 29S precursor (Underwood and Fried, 1990) and similar phenotypes are seen with either a cold

sensitive mutation in L16 (Moritz et al., 1991) or when L1 is depleted (Deshmukh et al., 1993). This suggests that efficient processing of the pre-rRNA is dependent on at least some of the ribosomal proteins.

ii). Non-ribosomal proteins

Mutational analysis has revealed that several proteins having one or more RNA binding domains are essential for pre-rRNA processing. In most cases these proteins do not bind the primary transcript but are complexed with snoRNAs (described above) and hence constitute the protein component of the snoRNPs. For example, in yeast, pre-rRNA processing is impaired when strains carrying deletions in genes encoding NOP 1 (all snoRNAs), NOP 5 (all snoRNAs) , SOF 1 (U3 specific), GAR 1 (snR10, snR30) and SSB 1(snR10) are observed (Schimmang et al., 1989, Wu et al., 1998, Jansen et al., 1993, Girard et al., 1992, Clark et al., 1990). The focus of some current studies is to elucidate how many proteins are complexed with the snoRNAs and to define their roles in the RNP complexes (Baserga et al., 1997, Pluk et al., 1998)

Nucleolin is the best characterized non-catalytic protein found in the nucleolus. The gene for it has been cloned in man (Srivastava et al., 1989), rat (Bourbon et al., 1988), mouse (Bourbon et al., 1988b), hamster (Lapeyre et al., 1985), chicken (Maridor and Nigg, 1990), *Xenopus* (Caizergues-Ferrer et al., 1989), *S. cerevisiae* (Lee et al., 1992) and *S. pombe* (Gulli et al., 1995). The central domain of this protein contains four RNA recognition motifs that have been evolutionarily conserved in vertebrates (Bugler et al., 1987, Query et al., 1989). The C-terminal domain is unusually rich in glycine, arginine and phenylalanine residues and on its own has the ability to destabilize and unfold RNA secondary structure

(Ghisolfi et al., 1992). Sequence analysis coupled with *in vitro* cross linking experiments has shown that mouse and human pre-rRNA is bound at many sites (approx. 80 total) by nucleolin (Serin et al., 1996). and can be immunolocalized to the Miller Christmas trees (Ghisolfi-Nieto et al., 1996). Most recently, *in vitro* studies have shown that it is essential for the primary cleavage event in mouse (Ginisty et al., 1998). Nucleolin remains the only known non-catalytic protein to bind the pre-rRNA.

Shortly after, or concomitantly with transcription, the pre-rRNA is modified by methylation and pseudouridinylation with all the modifications occurring in the mature sequences (discussed later). The most highly conserved of these events is a dimethylation that occurs at the 3' end of the SSU rRNA (van Knippenberg et al., 1984). A methylase enzyme has been cloned from *S. cerevisiae* that performs this dimethylation (Dim 1p)(LaFontaine et al., 1994). A conditional allele for the DIM 1 gene was constructed and when this enzyme was depleted, processing at A1 and A2 was impaired such that no mature 18S rRNA was produced (Lafontaine et al., 1995). It was further shown in this study that the methylation event itself was not important but instead the presence of the methylase was. It was hypothesized that the methylase somehow interacts with the processing machinery to ensure that only functional rRNAs are allowed to mature.

3. Cis-acting signals

A variety of studies indicate that rRNA processing events in each of the spacers is essential for rRNA maturation and as a consequence, much recent attention has been placed on determining the signals within the spacers that direct these cleavages. Because RNA molecules in solution generally contain extensive secondary and tertiary structure, potential

signals can be either at the level of the primary sequence, or may exist as higher order structural elements. Phylogenetic comparisons have revealed a few potential conserved signals (discussed below), but for the most part these types of studies have been disappointing due to extensive sequence divergence in these areas. Nonetheless, these diverged sequences may fold into similar structures that are important for ribosome biogenesis (Brimacombe, 1984, Nazar et al., 1987 Gonzalez et al., 1990, Michot et al., 1993). For example, a RNA structural element such as a crucifix, can have similar protein binding characteristics irrespective of its sequence composition. The role of the spacers is unclear, but it has been proposed that they may act to bring the ends of the mature rRNAs into close proximity for processing reasons (Nazar et al., 1987) or may serve to act as a quality control mechanism during ribosome biogenesis (Lee et al., 1995, Good et al., 1997a).

Due to the high gene copy nature of rDNA, studies involving gene replacement are not feasible, hence, previous reports generally were limited to signals within the 5'ETS, as defined by *in vitro* processing systems. However, recently systems have been developed to study all aspects of rRNA processing in both *S. cerevisiae* and *S. pombe*. The first system reported for *S. cerevisiae* was based upon introducing mutagenic rDNA repeats as episomal copies that were "tagged" in the mature sequences (Musters et al., 1989). Hybridization analysis allowed the identification of plasmid borne transcripts to be differentiated from the chromosomally derived rRNA. Subsequent to this, several groups have reported the use of systems in which conditional mutations in pol I allow for rRNA transcripts to be produced solely from introduced rDNA copies that are transcribed by pol II. (Nogi et al., 1993, Chernoff et al., 1994, Venema et al., 1995). A system similar to the one reported by Musters

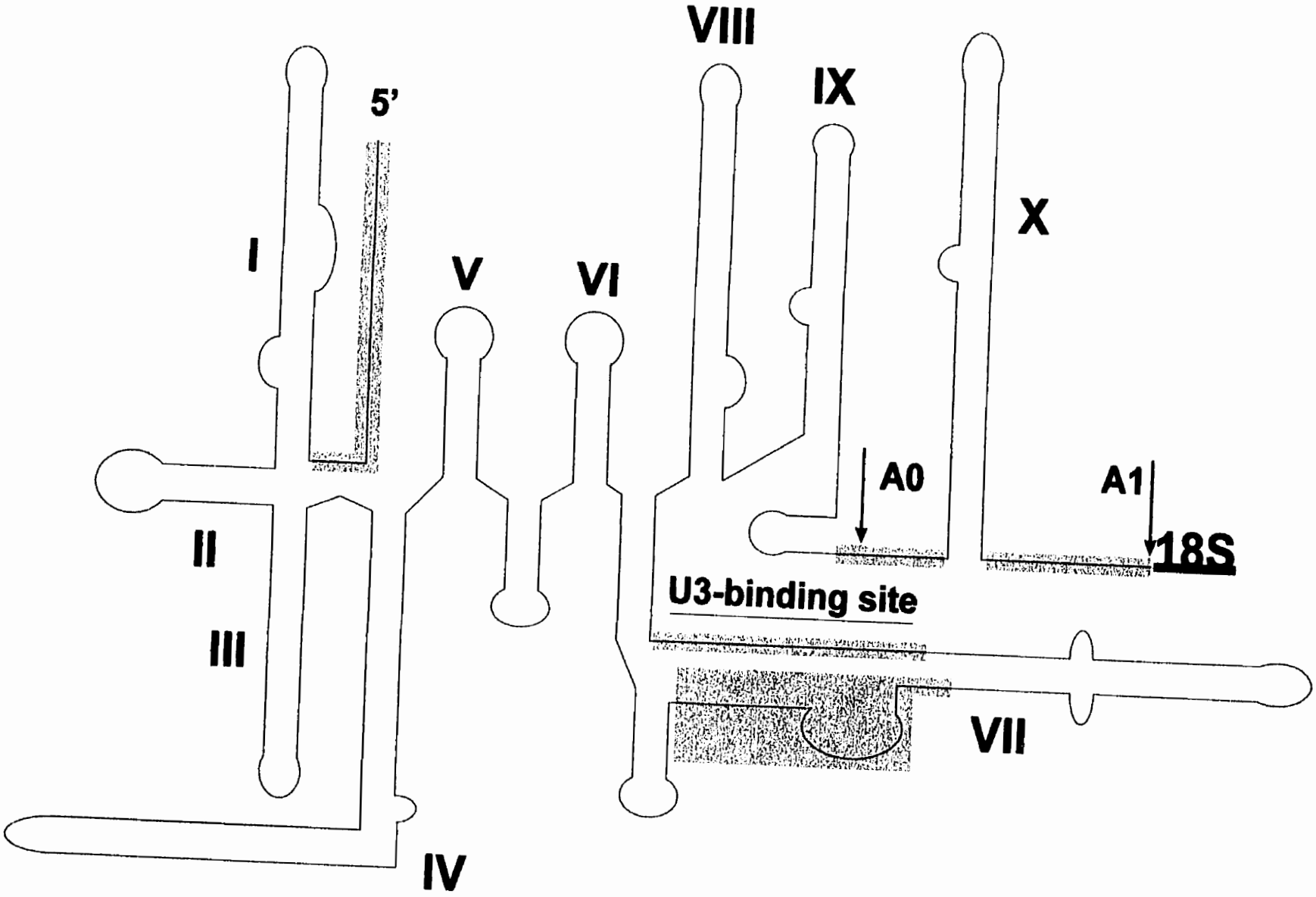
and co-workers has been developed for studying ribosome biogenesis in *S. pombe*. (Abou Elela et al., 1994, 1995).

a. 5' ETS

A secondary structure model has been proposed for this spacer from *S. cerevisiae* based on phylogenetic comparisons and chemical or enzymatic probing experiments performed on *in vitro* prepared RNA (Yeh and Lee, 1992). These studies suggested that the 700 base spacer is folded into ten helical domains (I-X) and the rest of the residues (10%) are distributed amongst five single stranded regions (Fig. 1.3) When five large deletion mutants, that ranged from 242 bp to 632 bp, were created and assayed for 18S rRNA production, no levels of 18S rRNA were detected (Musters et al., 1990). Unfortunately, all the mutations had at least 60 bp in common so conclusions about separate areas of this spacer cannot be drawn. The 5' 17 bases of the spacer are thought to be single stranded. Deletion of these bases with the first 11 bases of helix I resulted in no effect on 18S rRNA production (Lindahl et al., 1994). The single strand region opposite the U3 snoRNA binding site was assayed for its importance by deletion or substitution with the result that it too, had no effect on rRNA processing. Deletion (in whole or in part) of helices II and III have quite different effects. Helix II is essential for processing and 18S rRNA maturation, whereas deletion of helix III had minimal or no effect (van Nues et al., 1995). The helix most proximal to the 18S rRNA gene contains both the A0 and A1 processing sites and also has been examined in detail (Venema et al., 1995a). Removal of the top portion of this helix (not including either site) had no effect on processing and substitution of six nucleotides across the A0 site also had no effect. The six bases that immediately precede the 18S rRNA

Figure 1.3. Secondary structure model for the 5' ETS of *S. cerevisiae*.

The ten helical domains are number I-X and the five single strand regions are shaded. The positions of: the U3 snoRNA binding site, the A1 processing site, the A0 processing site and the 5' end of the mature 18S rRNA are indicated. The model was adapted from van Nues et al., 1995. have been conserved in yeasts (Venema et al., 1995a) and *Tetrahymena* (Good et al., 1997).


VIII
IX
X
5'
I
V
VI
A0
A1
18S
II
U3-binding site
III
VII
IV

Substitution of these bases was shown to have no effect on the production of 18S rRNA but may cause processing to proceed slower. To date, the best characterized cis-acting signal in this spacer is the U3 snoRNP binding site described earlier.

Unfortunately, *in vivo* systems have not been developed for higher eukaryotes, yet phylogenetic comparisons and *in vitro* generated data have revealed some interesting features in these organisms. A consensus folding model based upon comparative analysis and thermodynamic predictions for the large mammalian 5' ETS (3-4kb) has been proposed (Michot and Bachellerie, 1991). The comparative analysis was extended to include the 5' ETS (700b) of the amphibians *Xenopus laevis* and *Xenopus borealis*. It would appear that in spite of extreme sequence divergence and length differences, these spacers are similar in that they fold into four separate domains of secondary structure.

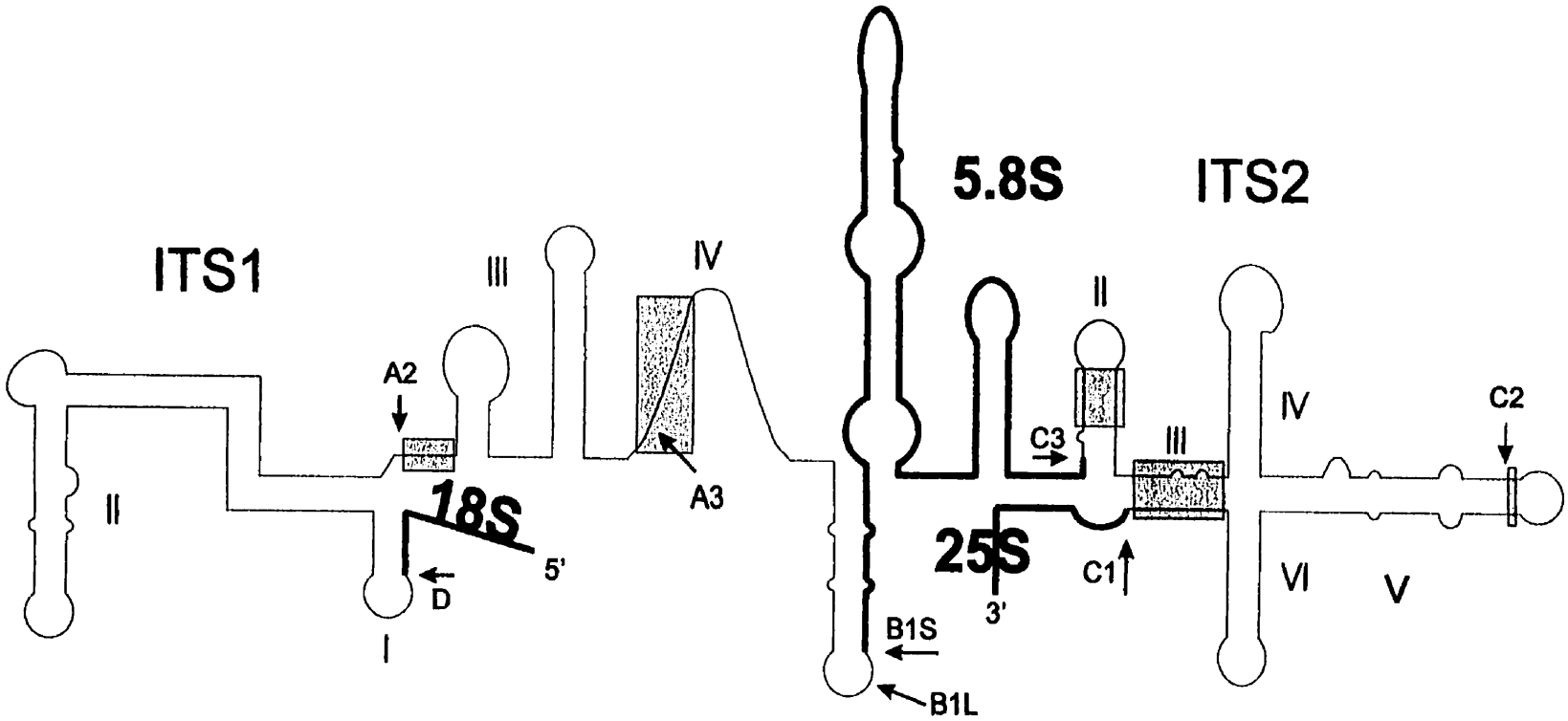
The 200 nucleotide segment immediately downstream of the primary cleavage site is approximately 85% conserved in terms of sequence in mammalian species and the essential bases that contain the processing signal for this event are within the first 120 nucleotides in the mouse (Craig et al., 1987, Craig et al., 1991). Similarly, the *Xenopus* species have a frog-specific 120 nt conserved element that is essential for processing (Mougey et al., 1993a). More strikingly, in all vertebrates the first eleven of the 120 nucleotides are conserved. *In vitro* studies have revealed that the mouse processing machinery can recognize *in vitro* derived frog 5' ETS sequences and perform the primary cleavage event. Further to this, the 120 nt sequence is sufficient to form the 20S processome complex described earlier. This suggests that the signals and/or the processing machinery that recognizes them has been conserved in higher eukaryotes.

b. ITS1 signals

As in the case of the 5' ETS, a secondary structure model for the yeast ITS1 sequences has been proposed based on comparative analyses and direct chemical and enzymatic probing of *in vitro* derived transcripts (Yeh et al., 1990a) (Fig. 1.4). This spacer appears to fold into five regions of secondary structure with significant single stranded regions towards the 3' end. Initial studies showed that complete replacement of this spacer with those from other yeasts, closely related to *S. cerevisiae*, did not affect processing, which suggested that the cis-acting signals were conserved among yeasts (van Nues et al., 1994). Close inspection of the sequences revealed that there were three areas of conserved sequence : the loop end of region II, a four base sequence (ACAC) within region III, and an eight base sequence within region IV. The sequences in regions III and IV lie immediately 3' and encompass the A2 and A3 sites respectively. When the conserved area of region II was deleted, no effect was seen. However, when the whole region was deleted there was a significant growth defect in the cells harboring this deletion. Further studies dissected the important sequences at the A2 site. Deletion constructs were made that removed either the six nucleotides 5' to the ACAC sequence, the sequence itself, or the six nucleotides downstream (Allmang et al., 1996a). This study showed that the 5' sequences were not important and surprisingly, the sequence itself was not essential, but deletion or substitution of the downstream sequences severely diminished A2 processing. It is interesting to note that removal of the 3' AC pair from the quartet caused the A2 site to be repositioned to an upstream ACAC (Allmang et al., 1996a). This suggests that this sequence may be recognized by the processing machinery. When regions IV (containing the A3 site) and V were deleted,

Figure 1.4. Secondary structure model for the *S cerevisiae* ITS1, 5.8S rRNA and ITS2 region of the pre-rRNA.

The model includes the ITS1 5.8 rRNA and ITS2 sequences. The position of the mature 18S, 5.8S and 25S rRNAs are indicated. The positions of the known processing sites are indicated. The helices for each of the ITS regions are numbered and the areas that were shown to be critically important for processing are indicated by shading. The model was adapted from van Nues et al., 1995.


ITS1
5.8S
ITS2
III
IV
II
A2
A3
C3
III
IV
C2
II
18S
5'
25S
3'
C1
VI
V
D
I
B1S
B1L

processing was again severely inhibited, but when either was present, processing occurred normally (van Nues et al., 1994). Mutation of the sequences that contain the A3 site (putative RNase MRP cleavage site) had no effect on growth (Henry et al., 1994). However, when combined with the above mutation that alters the sequence downstream of the A2 site, processing is not only affected within this spacer but also at A0 and A1 (Allmang et al., 1996b).

A very recent study was undertaken to determine a consensus core secondary structure for ITS1 from a number of unrelated organisms (Lalev and Nazar, 1998). Using updated computer algorithms, a core secondary structure appears to have been conserved in very distant organisms. For example, the small ITS1 of *T. vaginalis* (86 nt), and the much larger *S. pombe* ITS1 (421 nt), fold into a similar structure consisting of one major helix which is flanked on either side by much smaller helices that are adjacent to the mature ends of the 18S and 5.8S rRNAs. Enzymatic probing of *in vitro* derived ITS1 sequences from *S. pombe* was consistent with the computer predicted model for this region. In light of these results, it will be interesting to see if cis-acting signals that are conserved within ITS1 will be identified.

c. ITS2

Structural modeling of ITS2 from *S. cerevisiae* (Yeh and Lee, 1990b) has suggested that this spacer folds into six regions, and when compared with other yeasts, conserved sequences are found in regions II, III, IV and V (Fig. 1.4). Initial mutational analyses involved the replacement of this spacer with the sequences from other yeasts (van der Sande et al., 1992). Unlike the results obtained with ITS1, the only ITS2 replacement that

supported processing was from the most closely related *Torulaspora delbrueckii* and, although processing occurred, the generation time of cells carrying this mutation was significantly lengthened. van Nues and co-workers (1995b), performed a mutagenic survey study to determine which of the conserved regions were essential for processing. When the conserved bases in region II were included as part of a deletion, the amount of 25S rRNA was severely decreased and there was an appreciable increase in 27S precursor levels. The results of primer extension analysis showed that a small amount of mature 25S rRNA could be detected and the 5' terminus was in the correct position which would suggest that this region may affect the efficiency of processing. Deletions encompassing domain IV (except the conserved residues), or region VI were shown not to effect processing. The end of the helix in region V is in close proximity to the C2 processing site. Deletions or substitutions of the loop end had no effect but alteration of the last two pairs that precede the loop end could completely abolish processing of this spacer.

In all cases, the above studies reported that there was no effect on maturation of the 18S rRNA. In contrast to this, when the ITS2 region from *S. pombe* was deleted, thereby creating a similar situation to the prokaryotic 23S rRNA molecule, not only was 5.8S rRNA maturation reduced below detectable levels but approximately only 15% of the normal levels of the 18S rRNA were produced (Good et al., 1997b). The reasons behind the differences seen in *S. cerevisiae* and *S. pombe* is unclear.

d. 3'ETS

Sequence comparisons and deletion analysis of the 3' ETS of *S. cerevisiae* revealed that a highly conserved extended stem loop structure that forms immediately after the

mature 3' end of the 25S rRNA is necessary and sufficient for processing in this area (Veldman et al., 1981, Kempers-Veenstra, 1986) (Fig 1.5). Processing of this spacer has been most extensively studied in *S. pombe*. In this yeast, three processing sites have been identified within this structure and complete deletion or deletion of just the top portion of the stem and loop inhibits processing and no mature 25S rRNA is produced (Melekhovets et al., 1994). This study also revealed that processing of ITS2 was inhibited, as the levels of the 27S precursor were significantly elevated and no 5.8S rRNA was detected. In a follow-up study this hairpin structure was more extensively examined and it was shown that the top half of the stem was critical as removal of the loop end had no effect but structural disruption of the stem abolished 5.8S and 25S rRNA production (Hitchen et al., 1997). When the structural integrity was restored in these mutants by introducing compensatory base changes, processing also was restored. Additionally, mutations that affected the lower half of the stem did not have a negative effect on processing. The results of gel mobility shift competition assays correlated with the *in vivo* data, in that, a mutation that affected processing was unable to form RNP complexes. Together these results suggest that at least some of the cis-acting signals in this spacer are housed within the top half of the stem loop structure, and are recognized by one or more protein factors.

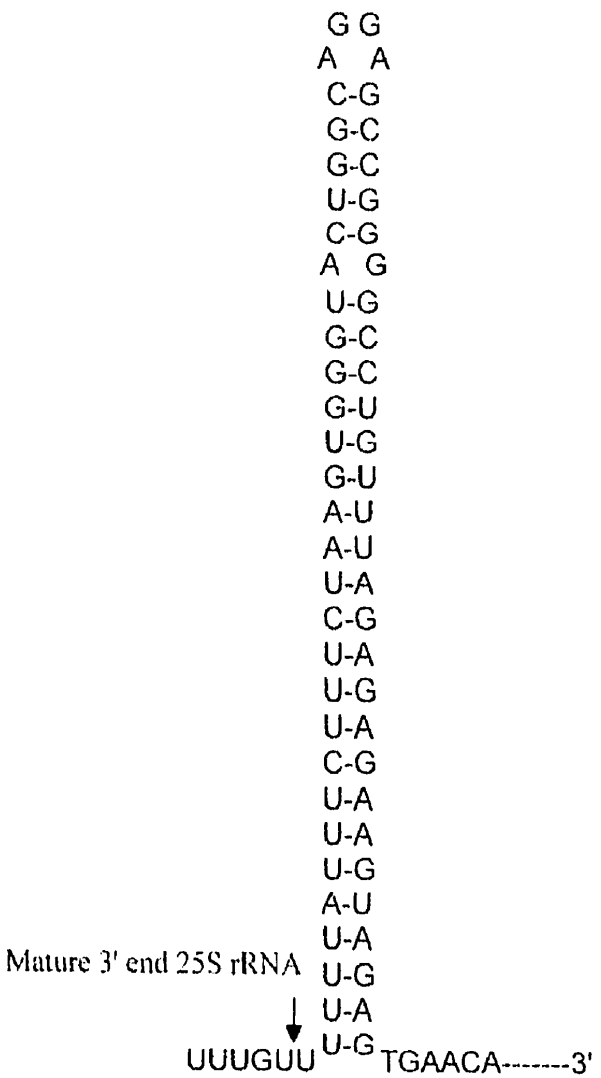
In another study it was shown that sequences downstream from this extended hairpin are not important for processing in *S. cerevisiae*, however, the structure itself is critical (Allmang and Tollervey, 1998). Whole or part deletion of the helix severely reduced the levels of 25S rRNA but did not alter the levels of 18S rRNA production. When further analysis was performed, results showed that removal of the structure had a specific

Figure 1.5. The structure of the 3' ETS from *S. cerevisiae* and *S. pombe.*

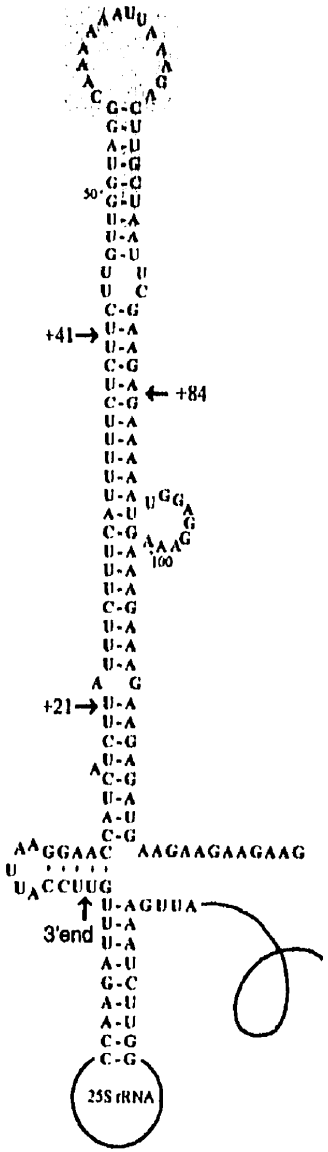
A. The structure of the 3' ETS region in *S.cerevisiae*. The position of the mature 3' end of the 25S rRNA is indicated. The structure was taken from Abou Elela et al., 1996.

B. The structure of the 3' ETS region in *S. pombe*. Processing cleavage sites are identified together with residue numbers. The position of the mature 3' end of the 25S rRNA is indicated. Bases that are critical for processing are indicated by the shaded region. The structure was taken from Melekhovets et al., 1994.

A.


G G
A A
C-G
G-C
G-C
U-G
C-G
A G
U-G
G-C
G-C
G-U
U-G
G-U
A-U
A-U
U-A
C-G
U-A
U-G
U-A
C-G
U-A
U-A
U-G
A-U
U-A
U-G
U-A
U-G
Mature 3' end 25S rRNA
UUUGUU
TGAACA-------3'

B.


+41
+84
+21
3'end
25S rRNA

inhibitory effect on the A3 cleavage site. Collectively the results demonstrate that the hairpin structure contains signals for 3' ETS processing and may contain signals for processing both ITS regions.

e. Signals within the mature sequences

There have been very few reports of cis-acting signals that are within the mature rRNA sequences. By extrapolation, signals or binding sites should be present in the mature rRNAs for any snoRNPs that bind these sequences and affect processing, such as U14. Although some of these trans-acting factors have been studied extensively, it remains unknown whether the signals are sequence or structure in nature. Recently, the first variable region (V1) of the 18S rRNA of *S. cerevisiae* has been shown to affect processing at the A0, A1 and A2 sites (van Nues et al., 1997). When mutations in V1 are analyzed, there is a build up of the 35S precursor and 18S rRNA levels are reduced. The reason behind this is unclear and it was postulated that a structure distortion introduced by this mutations may somehow block U14 from binding nearby sequences. Processing at both ends of the 18S rRNA may be dependent on signals close to the ends of this molecule. As described earlier, a pseudoknot structure exists that is formed three nt downstream of the 5' end of the mature sequence. Venema et al., (1995b) reported that the 5' terminus is always positioned three nt upstream of this structure regardless of what bases precede it. Therefore, it appears that this structure acts as a spacing signal that aids in processing at this end. Also, maturation of the 3' end of the 18S rRNA in mammals was shown to be dependent on a quartet of nucleotides that are one nucleotide from this end (Cavaille et al., 1996a). When this sequence or a putative U13 binding site were altered, a build-up of the 20S precursor was evident. Like

the SSU RNA, variable regions of the 25S rRNA sequence have been shown to affect processing of both ITS1 and ITS2 (Jeeninga et al., 1997). It is quite conceivable that further studies will reveal numerous additional signals located with the mature rRNAs.

4. Base modification

In addition to the many cleavage reactions that are performed in order to generate the mature termini of the rRNA genes, a number of nucleotides are modified by either the addition of methyl groups or by the conversion of uridine into pseuduridine (psi) (reviewed by Maden and Hughes, 1997, Tollervey and Kiss, 1997). In vertebrates, approximately 115 bases are methylated (105, 2'-O- ribose, 10 base methylations) and there are about 95 psi residues, whereas *S. cerevisiae* has fewer modifications, (55 2'-O-ribose, 10 base methylations and 45 psi residues) (Maden et al., 1995). The modifications occur on the nascent transcript, generally before transcription is complete. All known modifications are contained within the mature core rRNA sequences (Bachellerie and Cavaille, 1997).

Because there are so many 2'-0-ribose methylations, it was postulated that there should be a common sequence or structure within the rRNA that would signal which bases were to be modified but neither of these was found. Since the late 1980's, an increasing number of snoRNAs were isolated that had the following common features: 1) an association with fibrillarin (Cafferelli et al., 1996), 2) the presence of two evolutionarily conserved sequences (termed boxes C and D) and 3) the presence of complementarity to the mature rRNAs (10-22 nts) (Bachellerie et al., 1995). These molecules were without an assigned function until it was reported that the rRNA ribose methylation is guided by this family of snoRNPs (Kiss-Laszlo et al., 1996, Cavaille et al., 1996b). These studies

demonstrated that the antisense element of the snoRNA pairs with the corresponding region of the pre-rRNA. This pairing results in the conserved box D of the snoRNA being positioned exactly five nt upstream of the residue to be methylated. It is suggested that the methyl-transferase recognizes the box D element and then modifies the appropriate base (Kiss-Lazlo et al., 1996).

Similar to methylation, a class of snoRNAs is thought to guide the formation of psi residues. This family of snoRNAs has four common features: 1) two conserved sequence motifs termed boxes H and ACA, 2) an association with the Gar 1p protein 3) a shared common core secondary structure and 4) have short complementary regions to pre-rRNA (Tollervey and Kiss-Lazlo, 1997). A temperature sensitive mutation in the Gar 1p protein of *S. cerevisiae* inhibits psi formation of the rRNA (Bousquet-Antonelli et al., 1997) and Ganot and co-workers (1997), have shown that at least two of the H/ACA snoRNPs direct psi formation by interaction through the complementary sequence regions. Therefore, in a similar fashion to the methylation guide snoRNAs, this class recognizes a signal within the rRNA and directs the appropriate base to be modified. Most recently, an additional protein component of the H/ACA class of snoRNPs has been isolated which appears to be the putative psi synthetase (La Fontaine et al., 1998).

Despite over thirty years of investigation, the functional importance of rRNA base modifications remains unknown. Early studies indicated that methylation may play a critical role in ribosome maturation. For example, in mammalian cell cultures either methionine starvation, or substitution of methionine analogues resulted in a build-up of the 47S precursor and little or no mature rRNA production (Vaughan et al., 1967, Swann et al.,

1975, Caboche and Bachellerie, 1977). In contrast, Tollervey and co-workers (1993) showed that complete inhibition of methylation in *S. cerevisiae* did not inhibit ribosome biogenesis, however, the growth of the cells was severely impaired. Collectively these results suggest that global under-methylation of the rRNA has deleterious effects on growth and possibly ribosome function.

The modified nucleotides for the most part reside in the functionally important areas of the rRNA such as the decoding site and the peptidyl transferase center (Maden and Hughes, 1997). In addition, methylation is shown to have destabilizing effects on base pairing whereas psi residues have an increased capacity to pair and therefore may promote stronger pairing (Maden and Hughes, 1997). This suggests that the function of base modifications may be to fine tune the structure of the rRNA in the functionally important areas and allow for efficient ribosome function.

5. Processing as a quality control mechanism

Although a lot of information is known about the cis and trans-acting factors involved in the removal of the transcribed spacers, it is only recently that a function has been proposed for these sequences. In several studies, when maturation of the of the SSU rRNA or LSU rRNAs in *S. cerevisiae* were inhibited, maturation of the other was reported to be unaffected (Musters et al., 1989,1990, van Nues et al., 1993, Beltrame and Tollervey, 1995, Venema et al., 1995). Due to inefficient expression or lethality the above results are of a qualitative nature, yet they have lead to the hypothesis that processing of the rRNAs proceeds by a "split" maturation scheme where the LSU rRNAs mature independent of the SSU rRNA (Morrissey and Tollervey, 1995). Recent evidence to support this comes from

a study where growth was supported when the LSU and SSU rRNAs were produced *in trans*, which suggests that there is no need for maturation to be coupled (Liang and Fournier, 1997). However, two lines of evidence indicate that at least efficient processing may be dependent on formation of the whole transcript. The first of these comes from E.M. studies, were it was shown that processing (with the exception of the primary cleavage in mouse) does not occur until the transcript was complete (Miller and Beatty, 1969, Leger-Silvestre et al., 1997, Lazdins et al., 1997). Secondly, it is known that the primary transcript associates with proteins and forms a 90S particle in HeLa cells before any processing occurs (Kumar and Warner, 1972).

Recently, Nazar and co-workers have provided several lines of evidence that suggest that processing may occur in one large domain and that there is an interdependence in the maturation of the LSU and SSU rRNAs. This is based on an efficiently expressed vector system developed for *S. pombe* that allows for the quantification of plasmid derived transcripts (Abou Elela et al., 1994, 1995). Initially it was reported that alterations of sequences in the 3' ETS affected processing of ITS2 (Melekhovets et al., 1994, 1997). Insertion of the *Tetrahymana* ribozyme into the 25S rRNA also had severe detrimental effects on ITS2 processing (Good et al., 1994). More recently, no transcripts were detected when rDNA termination was abolished (Lee et al., 1995) and finally, when ITS2 was removed effects were seen on all processing steps (Good et al., 1997a). Combined, the results indicate that processing of the SSU and LSU rRNAs is very interdependent and, in general is sensitive to alterations in the structural domains of the whole molecule. As a result, it was hypothesized that cells have developed the transcribed spacers and such

schemes as "quality control" mechanisms which help to ensure that defective rRNA is not incorporated into active ribosomes (Good et al., 1997a).

F. Thesis overview

Despite being identified over fifty years ago, and the numerous studies which have been summarized in this introduction, many aspects of ribosomes and their biogenesis still remain poorly understood. When this work was initiated, the details of pre-rRNA processing pathways were restricted largely to the yeast, *S cerevisiae* and metazoans. Most of these details were determined from experiments where intermediates generated during processing were isolated through sedimentation centrifugation or observed utilizing hybridization studies. *S cerevisiae* has served as a model organism to study rRNA processing due to the ease with which it can be genetically manipulated and it's fast growth characteristics. *In vivo* model systems also were developed for this organism using episomal copies of rDNA which were tagged allowing for the specific detection of plasmid derived transcripts (Musters et al., 1989, Nogi et al., 1993, Chernoff et al., 1994). Results from this type of work showed that the primary transcript contained sequences elements in the 5' ETS and both ITS's that were essential. These studies also revealed that numerous trans-acting factors were involved in this process. At least one of these factors, the U3 snoRNP, was shown to be essential for viability. Further to this, the U3 snoRNP was shown to be essential for processing of the 5' ETS and could be cross-linked to the 5' ETS in both *S. cerevisiae* and metazoans (Tollervey 1987, Maser and Calvet, 1989, Stroke and Weiner, 1989, Beltrame and Tollervey, 1982). Studies that underlined the importance of the 5' ETS were first reported in 1990 (Musters et al.) when it was shown that a series of deletions in this spacer blocked the

formation of mature 18S rRNA. Unfortunately all the deletions had at least 60 bases in common and therefore it remained unknown if certain regions were more critical than others

In 1994, Abou Elela reported the development of a system to study rRNA processing in *S. pombe*. Similar to the systems developed for *S. cerevisiae* this system utilized episomal copies of rDNA which were tagged allowing for the specific detection of plasmid derived transcripts. However, this system was the first to allow accurate quantification of processing defects as at least 50 % of the rRNA generated was shown to be plasmid derived. Further to this some of the previously reported systems relied entirely on the plasmid derived transcripts for survival. As such, the episomally introduced rRNA did not have to compete with the chromosomally derived rRNA which may cause the cell to forgo some of its normal controls on rRNA production. Before the development of this system most of what was understood about pre-rRNA processing was based entirely on the results from studies with *S. cerevisiae* and it was unknown if the mechanisms were conserved in other organisms. Therefore, utilization of this system could provide a much needed perspective on such an important process.

The 5' ETS sequences from *S. cerevisiae* and *S. pombe* are approximately are 700 and 1300 bases long respectively and contain many structural features within them. Thus it was hypothesized that at least some of these structural features were important for ribosomal RNA maturation. Therefore this thesis was initiated to further the current understanding of rRNA processing ; more specifically the identification of structural elements that are essential for the removal of the 5' ETS in the fission yeast *S. pombe*. The majority of these studies employed the rDNA mutational analysis system developed by our group for *S. pombe* (Abou

Elela et al., 1994, 1995) that has successfully been used to study rRNA transcription termination (Melekhovets et al., 1994, 1997, Hitchen et al., 1997) , 5.8S rRNA function (Abou Elela and Nazar, 1997), rRNA processing (Good et al., 1997b), and ribozyme function (Good et al., 1994).

The experimental results obtained during the course of this work are presented in the four subsequent chapters. Chapter two provides an introduction to the mutational system developed by our group and presents background studies for the work performed in the following chapters. In order to study 5' ETS processing and subsequent 18S rRNA maturation, a neutral "tag", which allowed for detection of plasmid encoded derived transcripts by differential hybridization, was introduced into the 18S rRNA gene within the first variable region.

Chapter three presents an initial mutational survey of the 1312 b 5' ETS of *S. pombe*. Seven large deletions (350 bp - 52 bp) were constructed and assayed for both 18S rRNA and 5.8S levels in order to determine elements of the spacer which contain essential cis-acting signals. The results show that all areas of the spacer are important for 18S maturation except for the most 5' and 3' helices. A hypothesis that the 5' part of this spacer may be essential for processome complex formation is presented. Also included is a new strategy for PCR site directed mutagenesis.

In order to map more precisely the location of cis-acting signals within the 270 bases most proximal to the 18S rRNA, structural analysis of this area was performed and the results are presented in chapter four. The results obtained show this area to be base paired more highly than previously believed. When this new structure was compared to the same region

in *S. cerevisiae* it was shown that several features appear to spatially conserved including a seven nucleotide sequence that is part of the U3 snoRNP binding site previously identified in S .cerevisiae.

Based on the results obtained in chapter four, a series of 11 substitution mutations were created in order to pinpoint areas of the structure that are functionally important for 18S rRNA maturation and are detailed in chapter five. Regions that showed a significant affect were then further substituted in order to determine if the sequence, structure or both were the key features of these signals. During the course of this work it was shown that *S. pombe* has developed several different types of signals.

Chapter six presents a general discussion of the results obtained during the course of this work and provides directions for future studies.

Chapter 2. Plasmids and transcript specific tags.

A. Introduction

The trans-acting factors and cis-acting signals essential for rRNA maturation are best characterized for *S. cerevisiae* and this is especially true when considering the results that have been obtained *in vivo*. However, the systems that have been utilized have limitations. The first system reported used plasmid encoded mini-genes where the rDNA was replaced by a reporter gene and, essentially, only the pol I promoter and terminator regions remained (Kempers-Veenstra et al., 1984). Following this, a "tagged" rRNA system was reported (Musters et al., 1989). In this system, rRNA transcripts derived from plasmid borne mutant rDNA could be detected by virtue of oligonucleotide tags that were inserted into the 18S and 25S rRNA genes. Unfortunately, due to a low plasmid copy number, less than 0.5% of the total rRNA was plasmid derived and could only be detected through very sensitive hybridization analyses. Therefore, the results obtained were reduced to being qualitative in nature and subtle differences could not be detected. In 1991, van der Sande and co-workers discovered that rDNA units placed under RNA pol II promoters could be processed and assembled into functional ribosomes. This has led to the development of several "*in vivo* pol II" systems which in turn have furnished the majority of results that have been reported (Nogi et al., 1993, Henry et al., 1994, Lindahl et al., 1994, Venema et al., 1995a). These systems, while termed *in vivo*, are artificial and don't allow for alterations that may affect pol I to be assayed. Also in these systems, the rRNA is polymerized solely from pol II promoted copies of mutant rDNA which may force the cell to forego some of its normal control mechanisms to survive.

In the course of this thesis work, our lab developed an efficient *in vivo* mutational analysis system to study rRNA processing in *S. pombe* (Abou Elela et al., 1994, 1995). This system involves the use of a copy of the *S. pombe* rDNA repeat unit that has been "tagged" in the 5.8S rDNA gene with a four base insertion, cloned into the yeast shuttle vector, pFL20 (Fig. 2.1A). pFL20 has origins of replication for both *E. coli* and *S. pombe*, therefore mutations can be introduced into the rDNA and sufficient amounts of plasmid can be propagated in *E. coli* and then subsequently used to transform *S. pombe* (Fig 2.1A). In general, copy numbers of approximately 70 per cell are obtained and approximately 50% of the total rRNA is plasmid derived as detected by the altered electrophoretic mobility of the tagged 5.8S rRNA. This system has been used to study 5.8S rRNA function (Abou Elela et al., 1994, 1995, Abou Elela and Nazar, 1997), 3' ETS processing (Melekhovets et al., 1994), rRNA termination (Lee et al., 1995, Melekhovets et al., 1997), ITS2 function (Good et al., 1997a) and ribozyme function (Good and Nazar, 1994).

The central goal of this thesis is to examine structural elements in the 5' ETS of the *S. pombe* rRNA transcript that are essential for processing of this transcript and 18S rRNA maturation. At the onset of this work all reports from work performed with *S. cerevisiae* indicated that mutations which affected 18S rRNA maturation had little or no effect on the maturation of the LSU rRNAs. Therefore, in order to perform a mutational analysis of the 5' ETS it was necessary to "tag" the 18S rRNA sequences since "tags" in the *S. pombe* rDNA were restricted to the 5.8S and 25S rRNAs. This chapter describes the insertion of a new Pst-1 restriction enzyme site that allowed the specific detection of plasmid encoded 18S rRNA upon northern hybridization analysis. The introduction of the new tag coupled with the

earlier "tags" permit maturation of both LSU and SSU plasmid derived rRNAs to be assayed.

B. Materials and methods

1. Bacterial strains and growth conditions

Escherichia coli C490 (rec A-,rk-, mk-, thr-, leu-, met-) (Hanahan, 1983) was used as a host for the propagation of various plasmids. Bacteria were grown at 37°C in Luria-Bertanini (LB) medium (10g Bacto-tryptone, 5g Yeast extract, 10g NaCl per litre) or on plates (15g agar added per L of LB media). When large quantities of plasmid were required the cells were cultured in Terrific broth (TB) medium as originally described by Tartoff and Hobbs, 1987 (12g Bacto-tryptone, 24g Bacto-yeast extract, 4ml glycerol per 900 ml to which 100 ml of 0.17M KH2PO4, 0.72M K2HPO4 was added).

2. Yeast strains and growth conditions

Untransformed *S. pombe* strain (h-,leu 1-32, ura4-D18) (Heyer et al., 1986) was grown at 30°C in liquid YEA medium (0.5% (w/v) Bacto-yeast extract, 2% dextrose) with constant aeration. Cells transformed with pFL20 based vectors were grown at 30°C either in liquid YNB media (minimal media) [0.67% (w/v) yeast nitrogen base without amino acids (Difco), 2.0% dextrose, (supplemented with 200 mg asparagine and 80mg leucine per litre)] or on agar plates (1.5% (w/v)) with minimal media (Sherman et al., 1983).

When growth rate studies were performed the following conditions were used. Overnight cultures of *S. pombe* cells were grown in minimal medium supplemented with the appropriate amino acids at 30°C with constant aeration. When these cultures were in the logarithmic growth phase they were diluted to an $OD_{550nm} = 0.1$ in of 100 ml of pre-warmed media and placed at 30°C for further growth. 1 ml aliquots were taken every two hours and the OD_{550nm} was immediately assessed and recorded. This was done continually until the

culture reached OD_{550nm} of 1.2. The data was then plotted on a semi-logarithmic graph as optical density versus time and the doubling time of the cells was determined from the slope of the line generated during the logarithmic phase of growth.

3. PCR templates and cloning intermediates

During the course of these studies several intermediate vectors were created in order to facilitate PCR-based mutagenesis and subsequent cloning of the mutated sequences into the final vector (Figure 2.1). The plasmid pSp5'ETS270 encodes the 270 nucleotides of the 5' ETS most proximal to the 18S rRNA as well as the first 140 nucleotides of the mature rRNA sequence, cloned into the Xba 1 restriction endonuclease site in the phagmid pTZ19R (Mead et al., 1986).

p5'ETS18S5.8Cla contains the sequences from the Cla 1 restriction endonuclease site in the 5.8S rRNA gene upstream to the Spe 1 restriction endonuclease site in the pFL20 (refer to Fig. 1)cloned into pBluescript KS at the Xba 1 and Cla 1 restriction endonuclease sites and was used as an intermediate for cloning.

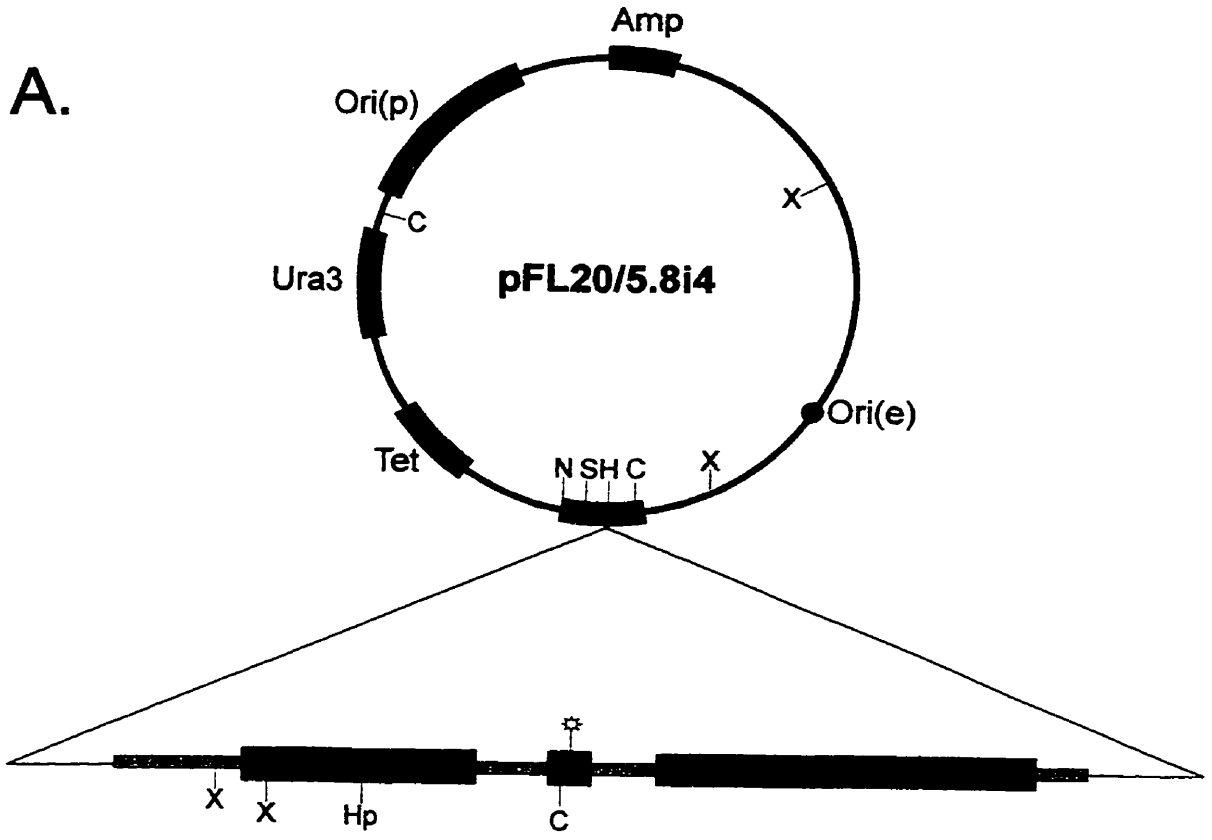
The shuttle vectors pFL20/Sp5.8i4 and pFl20/Pst18S5.8i4 (Fig. 2.1) used in this thesis are based on the pFL20 high copy yeast shuttle vector (Losson and Lacroute, 1983). Transformation of *S. pombe* ura4D strains to a Ura + phenotype and propagation of the vector is made possible by the vector-encoded *S. cerevisiae* URA 3 gene and an *S. pombe* origin of replication.

Figure 2.1 A diagrammatic representation of vectors utilized in these studies.

A. The *S. pombe* shuttle vector pFL20/5.8i4.Our group previously cloned a rDNA repeat unit into the *S. pombe* shuttle vector pFL20 at a Hind III restriction endonuclease site (Abou Elela et al. 1994). The 5.8S rDNA gene was subsequently "tagged" (*) the with a four base insert that allows electrophoretic detection of plasmid derived 5.8S rRNA transcripts and the insertion was shown to be neutral in all aspects (Abou Elela et al. 1995). Essential features of this vector are marked: Ori(e) = *E.coli* origin of replication, Ori(p)= *S.pombe* origin of replication, Ura= *S. cerevisiae* URA 3 gene, Amp= ampicillin resistance gene, and Tet= tetracycline resistance gene. Several restriction enzyme sites utilized in these studies are also marked: X= Xba 1, N= Not , C= Cla 1, S= Spe, Hp=Hpa 1 and H= Hind III.

B. p5'ETS18S5.8Cla contains the sequences from the Cla 1 restriction endonuclease site in the 5.8S rRNA gene upstream to the Spe 1 restriction endonuclease site in the pFL20 backbone cloned into pBluescript KS (Pharmacia) at the Xba 1 and Cla 1 restriction endonuclease sites. This vector was used as an intermediate for cloning.

C. The plasmid pSp5'ETS270 contains the 270 bases of the 5' ETS most proximal to the 18S rRNA as well as the first 140 bases of the mature gene, cloned into the Xba 1 restriction endonuclease site in the phagmid pTZ19R (Mead et al.1986). This vector was used initially to sequence this area as well as a template for mutagenesis studies.


A.
Amp
Ori(p)
X
C
Ura3
pFL20/5.8i4
Ori(e)
Tet
N SH C
X
X
X
Hp
C


B.
X/S
N
X
X
Hp
C
p5'ETS18S5.8Cla


C.
X
X
pSp5'ETS270

4. DNA preparation and analysis.

a. Polymerase Chain Reaction amplification and site-directed mutagenesis

Mutagenesis was performed using the polymerase chain reaction (Mullis et al., 1986) to introduce changes and amplify the resulting DNA. The reactions were performed in buffer containing: 50 mM KCl, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 0.1% TritonX-100, 200μM of each dNTP, 100 mg/ml BSA, 50 ng of each primer, 50 ng of template DNA and 1 unit of *Taq* polymerase. The reactions were performed using a thermocycler (LKB Gene ATAQ Controler, Pharmacia, Uppsala, Sweden) programmed with an initial denaturation step at 95°C for five minutes, followed by 33 cycles of denaturation at 95°C for one minute, annealing at an empirically determined temperature in the range of 40-55°C for one minute and extension at 72°C for one minute. A "tag" containing a new Pst I restriction endonuclease site was introduced into the 18S rRNA sequence by using the "megaprimer" technique (Sarkar and Sommer, 1990) (Fig2.2) with the extended annealing time modification described by Good and Nazar, (1992). The mutant PCR-1 product was electophoretically purified, extracted from an agarose gel by homogenization then subsequently used in a second reaction, to extend the initial product.

b. Purification of DNA fragments by agarose gel electrophoresis

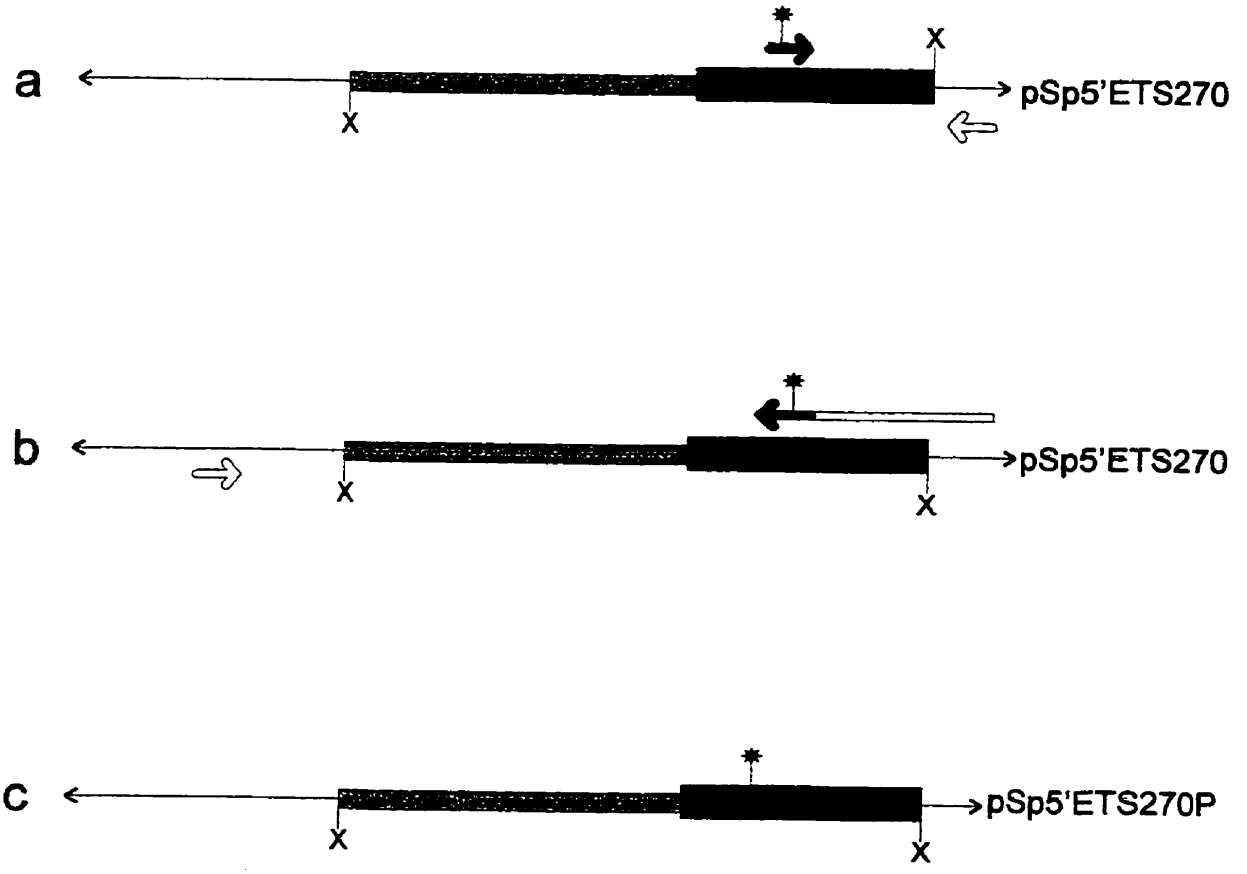
DNA was fractionated in agarose (Bio Shop, Canada) usually 1% (w/v) containing 0.5 X TBE buffer. Electrophoresis was performed at 33V/cm. Prior to loading, the reaction samples were mixed with a 1/10 volume of stop solution (50% (v/v) glycerol, 0.1 M EDTA (pH 8.0), 1% (w/v) SDS and 0.1% (w/v) bromophenol blue). After electrophoresis, gels were stained in a bath of ddH_20 containing 0.5 μg ml^{-1} EtBr for 15 minutes, destained in ddH_20

Figure 2.2 Introduction of a new Pst 1 restriction endonuclease site in the V1 region of the 18S rRNA sequence using "megaprimer-based" PCR-directed mutagenesis.

(a). In the first step a fragment of DNA was amplified using a mutagenic primer (CTAAGTATACTGCAGGTTGTACTGTG) which contains substitutions that generate a new Pst I restriction endonuclease site (closed arrow) and a non-mutagenic primer (open arrow). The resultant fragment contained the mutation, the 3' Xba 1 restriction endonuclease site and some vector-derived sequences.

(b).The fragment generated in reaction (a) was gel purified and used as a "megaprimer" in a second reaction with an upstream non-mutagenic primer (open arrow). This reaction extended the "mega-primer"sequence to include the 5' Xba 1 restriction endonuclease site and some additional 5' vector sequence.

(c). The product from reaction (b) was gel purified, cleaved with Xba 1 restriction endonuclease, and cloned into the Xba 1 site in pTZ19R. This generated the plasmid designated pSp5'ETS270P containing a new Pst 1 restriction endonuclease site.


a
X
X
pSp5'ETS270
b
X
X
pSp5'ETS270
c
X
X
pSp5'ETS270P

for at least five minutes and visualized using a hand-held, long wave-length UV illuminator. Images of DNA were visualized with long wave UV light (310 nm) with a transilluminator and were captured using a Gel-Doc camera system (Bio-Rad Laboratories, CA). Where appropriate, images were analyzed using Molecular Analyst®/PC software version 1.0 (Bio-Rad Laboratories, CA). Bands containing the desired DNA were excised using a scalpel and the DNA was recovered by homogenization. The gel slices were placed in 400 µL of extraction buffer (0.3% SDS in 0.14M NaCl, 0.05M Na acetate) in a glass homogenizer with a Teflon pestle and crushed with eight strokes. An equal volume (400 µL) of phenol solution (946 ml 90% phenol, 210 ml m-cresol, 135 ml ddH_20, 1.3g 8-hydroxyquinoline) was added to the suspension and the mixture was homogenized with another four strokes. Aqueous and phenol phases were separated by micro-centrifugation (12,500 g, three minutes)(Model 235B, Fisher scientific, Fairlawn N.J.). DNA solutions were concentrated by adding 2.5 volumes of salted EtOH (10 ml 3M K acetate to 240 ml 95% EtOH) followed by 24 hrs of incubation at -20°C. Samples were then centrifuged at 12,000 rpm for three minutes. at room temperature. The supernatant was decanted and pellets were dried in a Speed-Vac concentrator (Savant). The pellets were then resuspended in ddH_20 to a concentration of at least 100 ng/μl. DNA concentration was determined by reading absorbance at 260 nm on an LKB Biochrom Ultrospec II spectrophotometer. Concentration was assessed according to the relationship, $A_{260nm} = 1$ corresponds to 50 $\mu g\ ml^{-1}$ dsDNA (Maniatis et al., 1982).

c. Ligation and subcloning of DNA fragments

Cloning experiments involved the ligation of DNA (insert) fragments with either blunt

or cohesive ends of linearized plasmid DNA vectors. All ligations were performed at room temperature for four hours using a vector to insert ratio of 3.0 in a 20 μL volume containing two units T4 DNA ligase in 1 X ligase buffer [25 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP]. PCR fragments were either digested with an appropriate restriction endonuclease and ligated into pTZ19R cleaved with the same enzyme or directly ligated into Sma I (blunt) cleaved pTZ19R in the presence of Sma I endonuclease, as described by Liu and Schwartz, (1992). Restriction enzymes from various commercial sources (eg. Pharmacia Biomedicals, Uppsala Sweden, Bethesda Research Laboratories, Bethesda MD. New England Biolabs, Beverley MA etc.) were used to digest DNA (plasmid, genomic and PCR products). Digests were carried out under conditions of ionic strength and temperature recommended by manufacturer, using about one unit of restriction endonuclease per μg of DNA. In all cases, ligation products used to transform *E. coli* C490 to ampicillin resistance with 10 μL of the ligation reaction volume.

d. Bacterial cell transformation

Competent *E. coli* C490 cells were prepared as outlined by Chung and Miller, (1988) and Chung et al., (1989). A single colony from an LB plate was used to inoculate a 2.5 ml overnight culture which was incubated at 37°C. The culture was then subcultured 1:100 in LB+ 20mM $MgSO_4$ and grown to OD_{590} = 0.4-0.6. The cells were collected by centrifugation in a JA20 rotor (Beckman, Palo Alto, CA) at 5,000 rpm for five minutes at 4°C, gently resuspended in 0.4 volumes of TFB1 (30 mM KAcetate, 100 mM KCl, 10mM $CaCl_2$, 50 mM $MnCl_2$, 15% glycerol), incubated on ice for five minutes and again collected by centrifugation at 5,000 rpm. The pellet was then resuspended in 0.04 volumes of TFBII (10

mM MOPS, 75 mM $CaCl_2$, 10 mM KCl, 15% glycerol) incubated on ice for one hour and 100 µl aliquots were stored at -70°C.

The competent C490 cells were transformed to ampicillin resistance by the addition of 0.5 volumes of a ligation reaction mixture to an aliquot of frozen competent cells and incubation on ice for approx. 40 minutes The cells were then heat shocked at 42°C for 90 second and placed at room temp. for ten minutes This was followed by the addition of 600 ml of LB and further incubation for 20 minutes at 37°C with gentle shaking for 20 minutes At this time an appropriate amount (usually 50 µl) was plated onto LB plates containing 100 µg/ml ampicillin.

e. Colony hybridization analysis

E. coli transformed with ligation mixtures were screened for plasmids carrying inserts by colony hybridization techniques (Maniatis et al., 1982). Colonies were picked from transformation plates and replated onto LB-Amp plates using a grid pattern. The bacteria were then grown overnight at 37°C. A circular piece of filter paper (the size of the plate) (Whatmann 541, Springfield Mill, England) was placed carefully on top of the colonies and the colonies were transferred to the filter when it was lifted. The filter was next placed immediately onto absorbent material that had been pre-soaked with 0.5 M NaOH and incubated for eight minutes. This was repeated and the filter was transferred to Whatmann 3mm paper that had been presoaked with 0.5 M Tris-Cl/ 1.5 M NaCl and again incubated for eight minutes to neutralize its' base. After this was repeated, the filters were allowed to air dry, washed twice in 95% EtOH and again air dried before being baked at 80°C for one and a half hours.

Colony filters and Northern blots were probed with either radioactively labeled oligonucleotides or dsDNA. DNA was labeled by random-primed incorporation of [α^{32}P] dCTP (Feinberg and Vogelstein, 1983) using random hexanucleotide primers synthesized on a Cyclone "plus" automated oligonucleotide synthesizer. DNA (50 to 200 ng in 2 μL ddH_2O) and primers (2 ~100 ng) were denatured (three minutes at 95°C), cooled on ice and added to 25 nM dATP, dGTP, dTTP , 10 μCi [α^{32}P] dCTP (3000 Ci/mM) and one unit of Klenow enzyme in a volume of 20 µL. The reaction was carried out for one hour at room temperature and stopped by heating at 65°C for ten minutes. Percentage incorporation of radioisotope into DNA was determined by spotting 1 μL of the reaction solution onto a strip of a thin layer chromatography sheet (Polygram CEL 300 PEI, Machery-Nagel & Co.) and fractionating by ascending chromatography using 0.75 M potassium phosphate (pH 3.5). Autoradiography was used to visualize incorporated and unincorporated isotope. In order to determine the specific activity of the probe, Cerenkov radiation was detected in a Pharmacia-Wallac 1209 RackBeta liquid scintillation counter and used to calculate the incorporation per ug of DNA.

When radioactively labeled oligonucleotides were used as probes, 10 pmol of oligonucleotide in T4 polynucleotide kinase buffer (50mM Tris (pH 7.6), 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine, 0.1 mM EDTA (pH 8.0)) were incubated in the presence of four units of T4 polynucleotide kinase (Pharmacia) at 37°C for one hour. Incorporation of isotope was determined as described above.

In all cases the blots/filters were placed into glass roller bottles and incubated at an appropriate temperature in a Hybaid™ mini oven MK II overnight with constant rotation in

a minimal volume of hybridization buffer. The hybridization buffers used are as follows: for ds DNA probes 50% formamide, 6X SSPE, 0.5% SDS, 5X Denhardts solution 100 ug/ul salmon sperm DNA ; and for oligonucleotide probes 6X SSPE, 2X Denhardts solution, 1% SDS and 100 ug/ul salmon sperm DNA. (Rose et al., 1990). The membranes/filters were pre-hybridized with buffer alone for four hours at which time the denatured probe was added.

After hybridization the membranes/filters were washed in 2X SSPE and 0.1% SDS twice for ten minutes. At this time the wash solution was changed to 0.2 X SSPE and 0.1% SDS and the wash temperature and duration was dependent on the probe that was used. At this time the membranes were placed on glass plates, wrapped with a plastic film and exposed to X-ray film for autoradiography for an appropriate time. Images of the autoradiographs were captured using the Gel-Doc system.

f. Isolation of DNA from *E. coli*

Initially small scale bacterial plasmid preparations ("mini-preps") were carried out as described by Birnboim and Doly, (1979) in order to screen positive colonies detected with colony hybridization. In these experiments, 1.5 ml of overnight 2 ml cultures, that included ampicillin, were transferred to a microfuge tube and pelleted at 12,000 rpm for ten seconds. The cell pellets were lysed by suspension in 100 µL lysis buffer (50 mM glucose, 25 mM Tris-HCl (pH 8.0), 10 mM EDTA). After incubation for five minutes at room temperature, 200 µL of 0.2 N NaOH, 1% SDS was added to each suspension and the tubes were inverted several times and incubated for five minutes at room temperature. The suspensions were mixed gently by vortex for ten seconds after addition of 150 µL ice cold 3M potassium acetate (pH 4.8) and stored on ice for ten minutes before being cleared by centrifugation for

two minutes. The supernatant was collected and deproteinized with an equal volume of phenol:chloroform:isoamylalcohol (25:24:1) and mixed vigorously by vortex. The aqueous and organic phases were then separated by micro-centrifuge for two minutes, nucleic acids in the aqueous phase were precipitated with cold 95% EtOH for two minutes on ice, and collected by microfuge for three minutes at 23°C. The pellet was dried in a Savant sample concentrator and resuspended in 50 μL of ddH_20 containing RNase A (Sigma Company, St. Louis, MO) at 20 μg/ml. The DNA was then analyzed by restriction endonuclease digestion and agarose gel electrophoresis described in section 4(b).

Large scale preparations were also made for sequencing, isolation of restriction endonuclease fragments for further cloning and yeast transformation. Density gradient centrifugation was used to purify the DNA as described Good and Nazar, (1995). A 100 ml overnight culture of bacteria in Terrific broth with ampicillin (100 μg /ml) was lysed by suspension of cells in 5 ml lysis buffer (50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA). The cell suspension was incubated for five minutes at 23°C. 10 mls of 0.2 N NaOH, 2% SDS was added , and the contents were mixed by gentle inversion and incubated on ice for ten minutes. After addition of 6.25 ml of sodium acetate pH 4.8, the solution was mixed vigorously and incubated on ice for ten minutes. The bulk of the bacterial chromosomal DNA and cellular debris were removed by centrifugation for 15 minutes at 10K rpm at 4°C. The supernatant was collected and nucleic acids were precipitated by addition of 0.6 volumes of isopropanol followed by a ten minutes centrifugation at 10K rpm, 15°C. After the supernatant was decanted, the pellet was dried and dissolved in 9.0 ml ddH_20. At this time, 1.012 g of CsCl was added per ml of the DNA solution. The mixture

was transferred to two polyclear centrifuge tubes (13 X 51 mm) (Seton Scientific, Sunnyvale CA) which contained 0.1 ml of ethidium bromide (EtBr) (10 mg/ml in water). The tubes were balanced and heat sealed before centrifugation at 45K rpm for ten hours in a Ti 65.2 rotor using a Beckman L7-55 (Beckman, Palo Alto, CA.) ultracentrifuge. The lower band, which corresponded to covalently closed circular plasmid DNA, was collected and the EtBr was extracted from the aqueous plasmid DNA solution by mixing (1:1) with isopropanol and saturated with 20X SSC (3M NaCl, 0.3M Na-Citrate). The phases were mixed by inverting the tube several times, the lower aqueous phase was transferred to another tube and the process repeated until all traces of color were gone. Two volumes of ddH_20 were added to the solution and the tube was filled (2.5 volumes) with 95% EtOH to precipitate the DNA at -20 °C. After one hour, the plasmid DNA was collected by centrifugation at 12,500g for ten minutes. Salt was removed from the DNA pellet with a 70% ethanol wash, the pellet was dried and resuspended in ddH_20 to a final volume of 1 μg/μl.

g. Determination of DNA sequences

Direct sequencing of plasmid DNA was carried out by the dideoxy chain termination method (Sanger et al., 1977) using the protocols and reagents from the ^{T7}Sequencing™ kit (Pharmacia Biochemicals). Plasmid (1-2 μg) was denatured with 8 μL 2N NaOH and 32 μL water for ten minutes at 23°C, neutralized with 7μL of 3M sodium acetate (pH 4.8) and 4μL ddH_20 and precipitated with 2.5 volumes of ethanol. After washes with chilled 70% ethanol, the pellet was dried under vacuum and dissolved in 10.0 μL ddH_20, 2.0 μL appropriate sequencing primer (20 ng) and 2 μL annealing buffer (1M Tris-HCl pH 7.6, 100 mM $MgCl_2$,

160 mM DTT). Annealing took place by incubation at 65°C for five minutes, followed at 37 °C for ten minutes and then at 23°C for at least five minutes.

Labeling reactions were carried out by adding to the 14 μL template-primer mixture: 3.0 μL of labeling mix containing 1.375 μM each dATP, dGTP , dTTP, 333.5μM NaCl, 5.0 μCi [α-^{32}P]dCTP and 0.5 units T7 DNA polymerase. The reaction was carried out at room temperature for five minutes; during this time, individual 2.5 μL aliquots of termination mixes (long or short) were prewarmed at 37°C. These mixes contained 840 μM of dGTP, dATP, dCTP, dTTP and 2.8 μM respective ddNTP in 40 mM Tris-HCl (pH 7.6) and 50 mM NaCl. Each termination reaction received 4.5 μL of the labeling reaction. Termination reactions occurred at 37°C for five minutes and were stopped by the addition of 5 μL stop mix (0.3% each bromophenol blue and xylene cyanol FF, 10 mM EDTA (pH 8.0) in 97.5% deionized formamide) and incubated on ice.

Labeled fragments from sequencing reactions were fractionated in denaturing polyacrylamide gels (7.6g acrylamide, 0.4 g bis-acrylamide, 48 g urea, 20 ml 5X TBE per 100 ml) of uniform thickness using 0.5 X TBE buffer (10X stock: 0.89 M Tris, 0.89 M Boric Acid, 25 mM EDTA) and the following settings: 35 W, 40 mA and 1800 V. The gel was then removed from the apparatus, fixed in 5% methanol, 5% acetic acid for ten minutes and washed in H_2O for ten minutes before being dried under vacuum on Whattmann 3MM paper. The dried gel was exposed to either XRP or XAR film (Eastman Kodak Company, Rochester NY.) overnight at -80°C and developed at an appropriate time.

h. *S. pombe* transformation by electroporation

pFL20 based plasmids carrying mutations in the rDNA sequences were used to

transform *S. pombe* cells electroporation as described by Prentice (1992). Cells were grown in YED medium (2% dextrose, 0.5% yeast extract) at 30°C to $OD_{550\,nm}$ of 0.7 with constant aeration. The cells were collected by centrifugation at 5K rpm (4°C) using a JA20 rotor (Beckman, Palo Alto, CA.). The pellet was resuspended in 50 ml of ice cold 1.2 M Sorbitol (Fisher Scientific) and collected as described above. After the wash was completed twice, the pellet was resuspended in 1 ml ice cold 1.2M Sorbitol. Transformation of the competent cells was performed by adding 200 µL of cells to a chilled electroporation cuvette (Bio-Rad laboratories) containing 10 µg of the appropriate transforming plasmid. The cell/DNA suspension was gently mixed and pulsed using an electroporator (Invitrogen corp, San Diego,CA) set at 1600 volts, 200 Ω and 50 µF. The cells were then diluted in 0.5 ml of ice cold 1.2 M Sorbitol. and 200 µL were plated onto minimal selective media (0.67% nitrogen base without amino acids, 0.5% potassium phosphate monobasic, 200 µg ml^{-1} asparagine, 80 µg ml^{-} leucine, 2% dextrose, 2% agar).Cells also were electroporated without transforming DNA and served as contamination controls. Plates were incubated for up to one week at 30°C. Single colonies were picked from the plates and streaked onto fresh plates to serve as growth stocks for subsequent nucleic acid extractions and other experiments.

The relative copy number of plasmids in yeast transformants were determined by dot blot hybridization. Wild type and transformed *S. pombe* cells were grown (0.8 A_{550nm}, 100 ml) with aeration at 30°C in minimal medium broth (Leupold, 1970), harvested by centrifugation, and whole cell DNA was extracted by the method of Hoffman and Winston, (1987). Total DNA was isolated from *S. pombe* cells using modified published methods (Hoffman and Winston, 1987). Overnight yeast cell cultures (grown in YNB medium and

appropriate supplements) were collected using a JA20 rotor (Beckman) (5K rpm, five minutes) and resuspended in 0.5 ml TE pH 8.0. Cells were transferred to a 1.5 ml microfuge tube and again pelleted for five seconds by microfuge. The supernatant was decanted and the pellet of cells was resuspended in residual liquid and lysed by vortex for two minutes with 0.3g acid-washed HC1 and baked (80°C) glass beads (0.5 mm diameter, Bioproducts Inc. Barysville, OK, 0.2 ml lysis buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA) and 0.2 ml phenol:chloroform:isoamylalcohol (25:24:1). A further 0.2 ml of T.E. buffer was added before the aqueous and organic layers were separated by microfuge (12, 500 g, three minutes). Nucleic acids were then precipitated by the addition of two volumes (about 1.0 ml) of 95% ethanol. After centrifugation (two minutes, at 12,500 g), the pellet was washed once with 70% ethanol before drying. The RNA was removed by digesting the pellet in 0.4 ml ddH_20 with 30 μg RNase A (Sigma) at 37°C for 15 minutes. The DNA was then precipitated by the addition of approx. 1.0 ml of 95% EtOH followed by centrifugation (12,500 X g, five minutes, 4°C). The DNA pellet was washed in 0.2 ml ice cold 70% ethanol, collected again by centrifugation and dried using a Speedvac concentrator. The dried pellet was suspended in 50 μL of ddH_20 and dissolved for five minutes at 37°C. The sample concentration was determined by spectrophotometry and the sample quality was evaluated further by agarose gel electrophoresis.

The DNA was dissolved in water, heated to 100°C, rapidly cooled on ice and 2 μg aliquots were manually dot blotted onto nylon membranes (Kafatos et al., 1979). The membranes were baked for two hours at 80°C. At this time the blots were hybridized with

either a plasmid specific probe prepared by the methods described in section 4(e) or with a 5S rRNA probe. To prepare the unlabeled 5S rRNA, whole cell RNA was isolated and fractionated on an 8% polyacrylamide gel as previously described (Nazar and Matheson, 1977). The RNA was labelled at the 3'-end with cytidine [3', 5'-^{32}P] biphosphate using T_4 RNA ligase (Peattie, 1979). The labelling reactions included 2-5 μg of 5S rRNA, in 10.5ul of T4 RNA ligase buffer (for a 5X stock: 250 mM HEPES pH 7.5, 16.5 mM DTT, 75 mM $MgCl_2$, 50% DMSO (v/v), 0.05 mg BSA/ ml) which were heated to 65°C for three minutes and cooled rapidly on ice. A 1.5 μl aliquot of 1.0 mM ATP, 0.5 μl of T4 RNA ligase (approx. five units) and 0.5 μl (approx. 25 uCi) of labelled pCp were added and the mixture was incubated on ice overnight. Incorporation was assessed by TLC chromatography as described earlier. Hybridization and wash conditions have been previously described in section 4(e). The washed blots were exposed to X-ray film to visualize the dots, the membrane was cut into equal pieces and the amount of bound isotope was determined by scintillation counting.

5. Expression characteristics of yeast transformants

a. Isolation and purification of cellular RNAs

For low molecular weight rRNA, whole cell RNA was prepared as described by Steele et al., (1965). Yeast cells were grown to an $OD_{550\ nm}$ of 0.4, transferred to 50 ml plastic tubes and harvested by centrifugation (JA20 rotor, 10K rpm ,three minutes). About 0.5 g cells were resuspended with 3 ml SDS buffer (0.3% SDS, 0.14M NaCl, 0.05 M sodium acetate (pH 5.1) in a 50 ml centrifuge tube and an equal volume of phenol solution (phenol containing 16% (v/v) m-cresol, 10% (v/v) water and 0.1% 8-hydroxyquinoline). The mixture

was incubated at 65°C for 30 minutes with intermittent shaking. After centrifugation (JA 20 rotor 10K rpm, ten minutes), the cleared aqueous phase was extracted with chloroform and the nucleic acids were recovered by precipitation at -20°C overnight with 2.5 volumes of EtOH. The RNA pellet was dissolved in 200 µL ddH_2O and the concentration was estimated spectrophotometrically. RNA concentration was determined by reading absorbance at 260 nm on an LKB Biochrom Ultrospec II spectrophotometer. Concentration was assessed according to the relationship, $A_{260nm} = 1$ corresponds to 40 μg/ml RNA (Maniatis et al., 1982).

For whole cell RNA analysis the RNA was isolated according to the protocol of Rose et al., (1990). From 100 ml cell cultures grown to an OD_{550nm} of 0.4 cells were rapidly harvested by centrifugation (JA 20 rotor, 10K rpm, three minutes) and resuspended in 0.625 ml ice cold LETS buffer (0.1M LiCl, 10 mM EDTA, 10 mM Tris-Cl (pH 7.4), 0.2% SDS). The cell slurry was transferred to a glass tube containing 2.75g acid-washed glass beads (Sigma) in 0.75 ml of phenol that was equilibriated previously with LETS buffer. The mixture was mixed by vortex top speed, by alternating 30 seconds of vortex steps with 30 seconds on ice for a total of six minutes At this time, an additional 1 ml of ice cold LETS was added to the mixture and the contents of the glass tube were transferred to a polypropylene centrifuge tube. Residual cells/beads/LETS solution remaining in the glass tube were rinsed with another 1 ml of LETS buffer which also was collected into the polypropylene tube. Aqueous and organic phases of the extract mixture were separated by centrifugation (JA20 rotor, 12K rpm, ten minutes) and the aqueous phase was then twice extracted with 3ml of phenol:chloroform:isoamyl alcohol (25:24:1). Finally, the supernatant

was transferred into a fresh tube and precipitated with 2.5 volumes of 95% EtOH containing 2% K acetate at -20°C for a least two hours.

b. Quantification of plasmid-derived 5.8S rRNA

To determine the relative amount of plasmid derived 5.8 S rRNA being produced in transformed cells, low molecular weight RNA was extracted from whole cells as described above and fractionated on 40cm x 24cm 8% polyacrylamide denaturing gels by electrophoresis using a constant wattage of 35W for approximately four hours The gels were stained with methylene blue (0.2% methylene blue in 0.2 M acetic acid, and 0.2 M sodium acetate) for ten minutes and constantly rinsed with ddH_20 until only the RNA remained blue. Images of the gels were captured using the (BioRad) gel Doc camera system and the amount of plasmid derived 5.8S rRNA as a percentage of the total 5.85 rRNA was determined using Molecular Analyst Software ver. 1.0.

c. Northern blot analysis of ribosomal RNAs and precursors

Whole cell RNA prepared as described in section 5(a) was fractionated in a 1.5 % Agarose/0.2 M formaldehyde gel buffered with 10 mM MOPS (0.02 M MOPS, 0.005 M NaAc, 0.001 M EDTA adjusted to pH 7.0 with acetic acid) as described by Rose et al., (1990). After electrophoresis at 50W for approx. three hours, the gels were washed in ddH_2O with several changes in order to remove the formaldehyde. The RNA was then blotted downward overnight (Chomczymski and Mackey, 1994) on a nylon membrane (Microseparations Inc., Westborough, MA) using 10X SSCPE (2.4 M Nacl, 0.3 M NaCitrate, 0.2 M KH_2PO_4, 0.02M EDTA adjusted to pH 7.2 with NaOH), rinsed in 2X SSCPE and baked at 80°C for one and a half hours After baking, the membranes were rinsed in ddH_2O

and stained with methylene blue stain (described above) and then destained in ddH_2O so that the RNA bands could be visualized clearly. Images of the membranes were captured using the Gel- Doc camera system. The membranes were subsequently hybridized, washed and exposed to X-ray film as described in section 4(e).

C. Results and Discussion

1. Sequence of the 5'ETS/18S rRNA region in *S. pombe*

As indicated, the goal of work presented in this chapter was to introduce a suitable marker within the 5' end of the 18S rRNA sequence that would allow plasmid derived 18S rRNA transcripts to be identified specifically. To facilitate both this and subsequent mutagenesis, an Xba 1 restriction endonuclease fragment encompassing the last 270 nucleotides of the 5' ETS and the first 164 bases of the mature 18S rRNA gene was subcloned from the plasmid encoded rDNA repeat into the Xba 1 site of pTZ19R (Fig 2.1C). To verify this construct, as well as previous sequence studies, the nucleotide sequence of this region was determined by direct plasmid dideoxy sequencing using the forward and reverse universal pTZ19R primers (Mead et al., 1986). A sequence alignment was performed with the equivalent sequence reported in the genebank, accession number Z19578. As can be seen in Fig.2.3, there is no variability within the mature 18S rRNA sequences however there are two areas that have deletions within the 5' ETS sequences. It is unclear if these changes represent strain differences or errors in the previously determined sequence.

2. Introduction of a Pst 1 restriction endonuclease site at the first variable region of the 18S rRNA.

The first variable region within SSU rRNAs is situated approximately 70 bases from the mature 5' end of these molecules (Dams et al., 1988). The variability that is seen between different organisms is attributed to both length differences in the stem as well as the number of bases that comprise the loop end (fig 2.4A). At the beginning of these studies there were no reports of studies involving this area. However, it was felt that the safest way to introduce

Figure 2.3 The nucleotide sequence of the Xba 1 restriction endonuclease fragment containing the last 270 bp of the 5' ETS and the first 140 bp of the 18S rDNA gene from *S. pombe.*

A. The Xba 1 fragment was cloned into pTZ19R and the sequence of this insert was determined by direct dideoxy nucleotide plasmid sequencing was performed (Sanger et al., 1977) The bold bases are nucleotides that were reported in the Gene Bank accession number Z19578 but are not present in the shuttle vector pFL20/5.8i4. The bases of the mature 18S rDNA sequence are underlined and the sequences shown in B are highlighted.

B. The sequencing autoradiograph presented shows typical sequencing reactions of the bases highlighted in A. The bases shown in parentheses indicate one of the regions that differs from the previously reported sequence. The sequencing gel is read from bottom (5') to top (3').

A

TCTAGACAAC CTCATATAAA ATTAATTTTT TAATTGAGGA ATAATGGGAA

ATTGTAAAAA AAAAATTTAG GAAGAATTTA TTTTCTTTTC TCTTTTTTTT

TTTTTTTCAG TTTCACCTTT GAGTAACTTT TGTTTTTGTT TTTTTCTTCC

TGATTTTGTT GGGAAGAAAA AAAATTAAAA ACGGTAGTGG TGTGTGAAAA

GTAAAAAGAA GTTTTAAAAA AAAACATTTT TTTATCTTCT TCTTTTCTGC

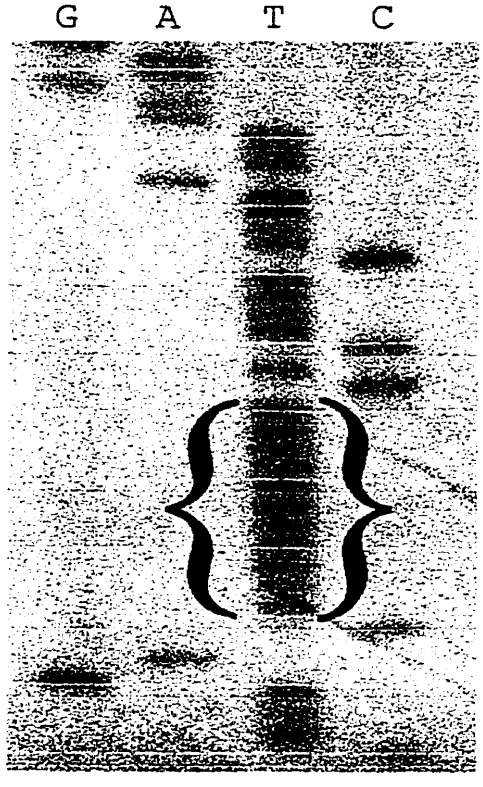
TTTTTGTCTC ACCTCTAACG ATAGTTACCT GGTTGATCCT GCCAGTAGTC

ATATGCTTGT CTCAAAGATT AAGCCATGCA TGTCTAAGTA TAAGCAATTT

TGTACTGTGA AACTGCGAAT GGCTCATTAA ATCAGTTATC GTTTATTTGA

TAGTACCTCA ACTACTTGGA TAACCGTGGT AATTCTAGA

B



a mutation into this area would be to maintain the structure as close to the natural state as possible (Figure 2.4B). Following the engineering of the "tag", it was reported, that base alterations which did not affect the structure were tolerated in *S. cerevisiae* and normal amounts of 18S rRNA were produced (van Nues et al., 1997). But, alterations that either affected the length of the helix, or the number of bases that comprise the loop had severe effects on 18S rRNA production. As discussed below, our data is consistent with this report in that base alterations of the V1 region of *S. pombe* did not have any harmful effects on 18S rRNA production.

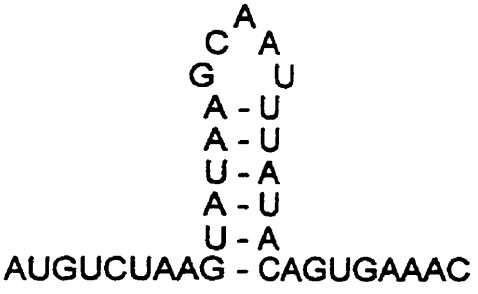
In order to disrupt the natural structure as little as possible, a new Pst 1 restriction endonuclease site was introduced (Fig. 2.4B) via the mega-primer approach (Fig.2.2) as modified with an extended annealing time (Good and Nazar, 1992). The resulting mutated and amplified DNA was then digested with Xba 1 restriction endonuclease and subcloned into pTZ19R. The new construct pSp5'ETS270 then was sequenced again to ensure that the mutation was correct and no unwanted alterations were introduced during amplification (results not shown). Due to the lack of convenient restriction enzyme sites, the Xba 1 fragment containing the new mutation was subcloned subsequently to construct a useful intermediate vector, p5'ETS18S5.8Cla (fig 2.1 B). In subsequent mutagenesis, the pCSX vector was cleaved with Not 1 and Hpa 1 restriction endonucleases and the Not 1/Hpa 1 fragment was used to replace the normal sequence in the pFL20/5.8i4 shuttle vector containing the 5.8S rRNA tagged *S. pombe* rDNA transcriptional unit. This gave rise to a new vector, pFL20/Sp18Pst5.8i4, which now contained tags that would enable detection of both LSU and SSU plasmid-derived rRNA transcripts.

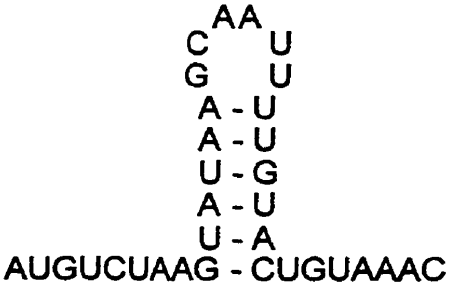
Figure 2.4 Putative secondary structure of the first variable region (V1)in the 18S rRNA.

A. The putative secondary structure of the V1 region of 18S rRNA for *S.cerevisiae* (left) and *S. pombe* (right) are shown.

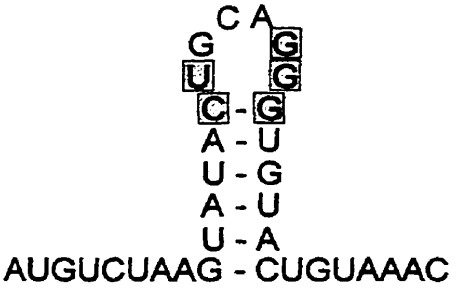
B. An estimate of the secondary structure for the V1 region when bases were altered to include a new Pst 1 restriction enzyme site. The substituted bases are shaded.

A.


A
C A
G U
A - U
A - U
U - A
A - U
U - A
AUGUCUAAG - CAGUGAAAC


AA
C U
G U
A - U
A - U
U - G
A - U
U - A
AUGUCUAAG - CUGUAAAC

B.


C A
G G
U G
C - G
A - U
U - G
A - U
U - A
AUGUCUAAG - CUGUAAAC

3. *In vivo* expression of the pFL20/Sp18Pst5.8i4 rRNA transcription unit

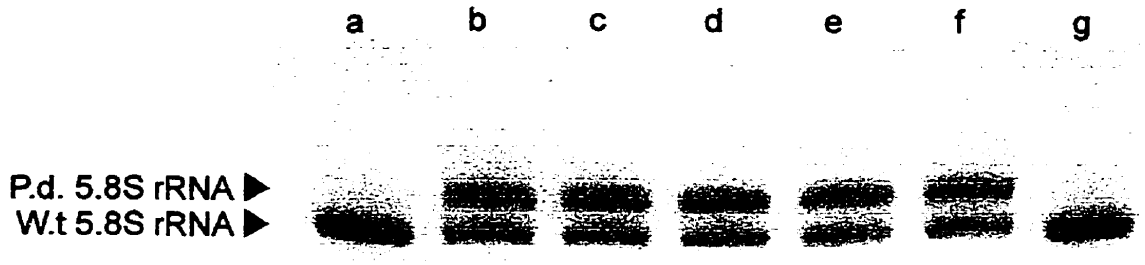
The pFL20/Sp18Pst5.8i4 plasmid and the vector from which it was derived, pFL20/Sp5.8i4, were used to transform *S. pombe* cells by DNA electroporation [section 4(h)]. Individual colonies were picked from the transformation plate and streaked for further growth. Cells from the streaks were then placed into liquid media and grown to an $O.D._{550}$ = 0.6-0.8 at which time both low molecular weight and whole cell RNA extracts were prepared. The low molecular weight RNA was fractionated on 8% denaturing polyacrylamide gels in order to distinguish the wild type (Wt) background 5.8S rRNA from the plasmid derived 5.8S rRNA. As shown in Fig. 2.5A, the introduction of the Pst 1 site within the 18S rRNA sequences did not have any effect on the production of 5.8S rRNA with an equivalent amount of the plasmid derived 5.8 rRNA being present in both the control RNA, (lanes b and f) and RNA extracted from cells that were transformed with the newly tagged 18S rRNA (lanes c, d and e) (Table 2.1). The whole cell RNA was fractionated on 1.2% agarose/formaldehyde gels using a MOPS-based running buffer. The RNA was then transferred by blotting overnight onto nylon membrane and stained with methylene blue in order to ensure an equal amount of RNA was applied to each gel lane (Fig. 2.5B left panel). The membranes subsequently were washed and hybridized with a labeled oligonucleotide probe (GTCACCCTGCAGTATAC) specific for the Pst 1 tag that was introduced into the 18S rRNA sequence. As shown in fig 2.5B (right panel), plasmid- derived 18S rRNA could be distinguished readily from the host cell rRNA by this method of differential hybridization as strong signals were generated only from RNA that was derived from cells transformed with pFL20/Sp18Pst5.8i4; lanes (a,b and c). It is important to note that there appears to be

Figure 2.5. ***In vivo*** **expression of plasmid-derived tagged rRNA in** ***S. pombe*** **transformed with pFL20/18Pst5.8i4.**

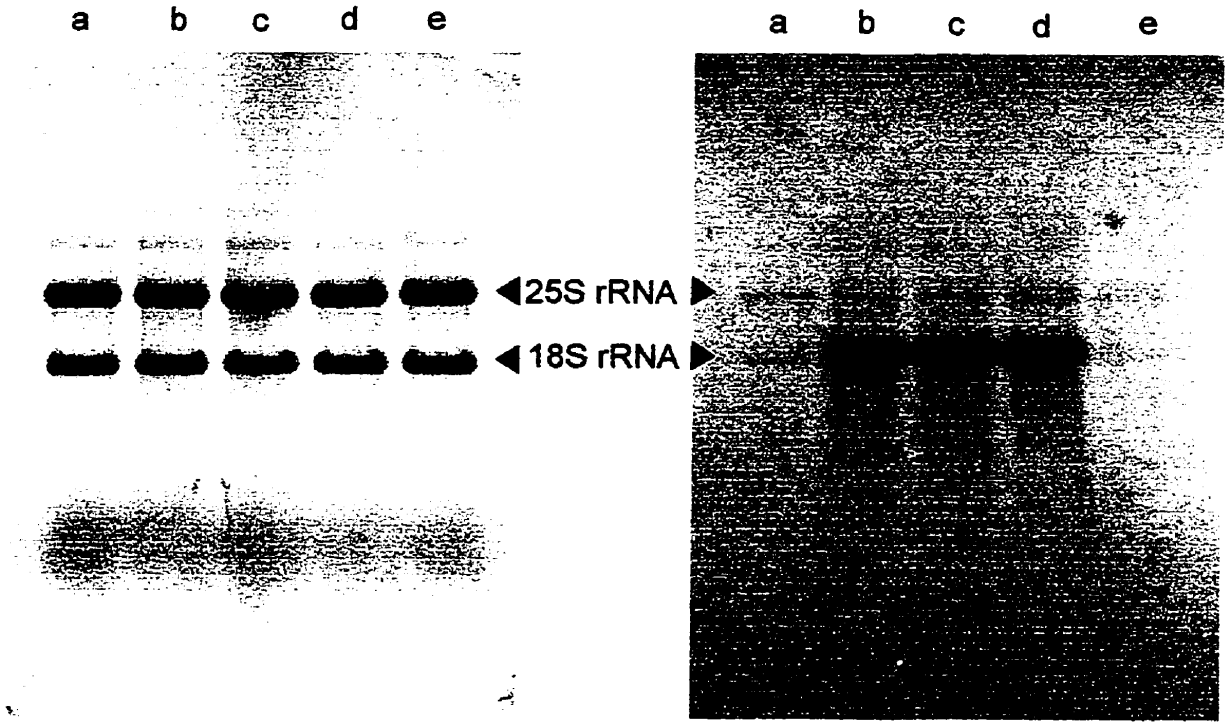
A. Expression of plasmid-derived 5.8S rRNA. Low molecular weight RNAs (20 ug) were prepared and fractionated on an 8 % denaturing polyacrylamide gel for four hours as described in Materials and Methods (2B6a). Lanes; a and g untransformed cells, b and f cells transformed with pFL20/5.8i4, and c,d,e cells transformed with pFL20/18Pst5.8i4. Plasmid-derived (P.d.) and wild type (W.t.) 5.8S rRNA species are indicated at the left.

B. Expression of plasmid-derived 18S rRNA. Whole cell RNA was extracted, fractionated on 1% agarose gels and transferred to nylon membrane as described in Materials and Methods 5(c). Left panel, methylene blue stained nylon membrane. Right panel, autoradiograph of the membrane in the left panel after hybridization with the Pst1 site specific oligonucleotide (GTCACCCTGCAGTATAC). Lanes a, untransformed cells, b, c, and d, cells transformed with pFL20/18Pst5.8i4 and e, cells transformed with pFL20/5.8i4. The positions of the 18S and 25S rRNAs are indicated.

A.



B.



some cross hybridization to the 25S rRNA sequences.

4. Plasmid copy number in *S. pombe* cells transformed with pFL20/Sp18Pst5.8i4

The plasmid pFL20/18Pst5.8i4 was going to be used extensively in subsequent experiments as a control vector. For this reason, it was necessary to establish a copy number for this plasmid. This, and subsequent copy number determinations would eliminate the possibility that effects observed in further mutational analyses were due to differences in copy number in various transformants. To determine the copy number, equal amounts of whole cell DNA from different transformants were blotted and hybridized with a plasmid-specific probe. As shown in Figure 2.6 (lower panel), this probe did not hybridize with DNA from untransformed cells (Wt) but indicated equal amounts of plasmid DNA in each sample of a transformed cell. To ensure that equal amounts of DNA were extracted from the cells and applied to the membranes, the blots also were hybridized with a 5S rRNA probe (Fig. 2.6 upper panel) and the relative radioactivity in this case was used to correct for sampling variations. As shown in Table 2.1, when so standardized against the host cell 5S rRNA gene, the analyses clearly demonstrated a comparable plasmid copy number in each case.

5. Growth rate of *S. pombe* cells transformed with pFL20/Sp18Pst5.8i4

To ensure that the introduction of the Pst 1 restriction endonuclease site into the 18S rRNA sequence did not have negative effects on growth, doubling times for transformants were determined. Yeast cells that were either transformed with pFL20/5.8i4 or pFL20/18Pst5.8.4 were grown as overnight cultures. When the cultures were in the logarithmic growth phase they were subcultured to an $O.D_{550} = 0.1$ and aliquots were taken every two hours and the optical density was determined. The values were plotted on

Figure 2.6. Determination of the plasmid copy number by dot blot hybridization analysis

DNA was extracted from either untransformed cells (W.T.), cells transformed with pFL20/5.8i4 (C1,C2) or cells transformed with pFL20/18Pst5.8i4 (P1-P3) and 2 ug aliquots were blotted onto nylon membrane as described in Materials and Methods [4(h)]. The upper panel is a representative autoradiograph of such a blot probed with ^{32}P-labeled 5S rRNA as a control for the amount of DNA spotted. The lower panel is a representative autoradiograph of such a blot probed with a plasmid specific DNA fragment as described in Materials and Methods [4(h)].

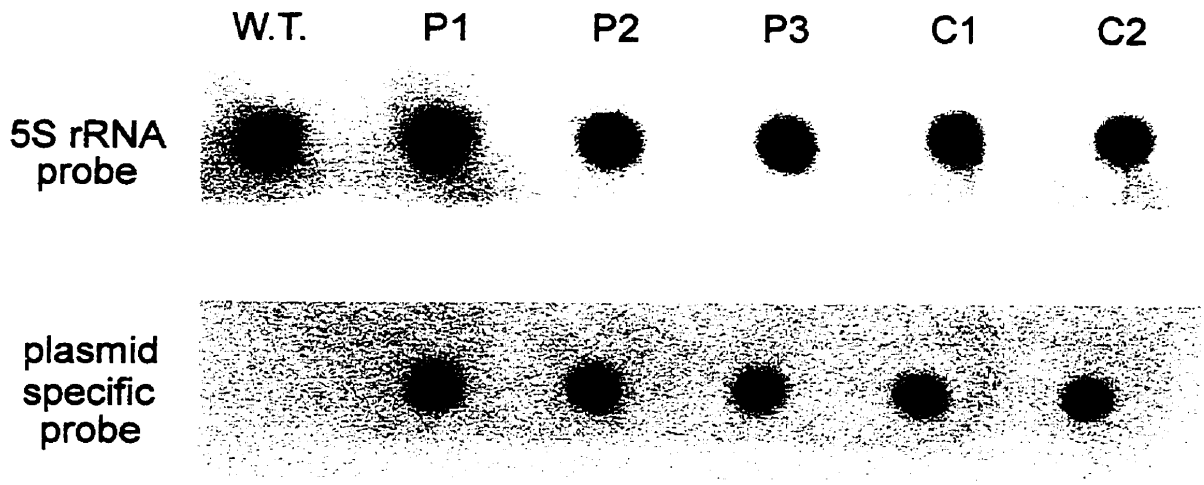

W.T.
P1
P2
P3
C1
C2
5S rRNA probe
plasmid specific probe

Table 2.1 Expression and growth characteristics of "tagged" *S. pombe* rDNA.

Plasmid	Plasmid derived 18 S rRNA[a]	Plasmid derived 5.8S rRNA[b]	Relative plasmid copy number[c]	Doubling time of transformants[d]
pFL20/5.8i4	none detected	52.1 +/- 1.2	74 +/- 2.6	4.3 +/- 0.2
pFL20/18Pst5.8i4	easily detected	52.3 +/- 1.1	74 +/- 1.2	4.3 +/- 0.1

a. Whole cell RNA was extracted and fractionated on a 1.2 % MOPs-agarose gel: the RNA was transferred to a nylon membrane and probed with a tag specific oligonucleotide. The hybridized membrane was then exposed to X-ray film for autoradiography.

b. Low molecular weight RNA was isolated from logarithmically growing cells, fractionated in 8% polyacrylamide gel, stained with methylene blue and band intensities were determined by densitometry. The values were determined from at least ten replicate experiments and the mean values are expressed as a percentage of the total 5.8S rRNA detected. The standard deviation is also included.

c. DNA was extracted from transformed *S. pombe* cells and manually spotted onto nylon membrane. The copy number reported is relative to the previously reported value of 74 for the control plasmid pFL20/5.8i4. The mean value was determined from five replicate experiments and the standard deviation is given.

d. Doubling times are given in Hrs, and the standard deviation determined from five replicate experiments is included.

semi-logarithmic graph paper and the doubling time was taken from the slope of the line generated. As can be seen in table 2.1, the introduction of the Pst 1 tag did not alter the growth rate of the yeast transformed with this plasmid when compared with cells in which the plasmid did not include the PST1 marker.

In order for this "tag" to be useful in the further studies, it was necessary to ensure that: 1) the plasmid encoded rRNA could be differentially detected from its wild type counterpart and 2) that the tag chosen was neutral and did not have deleterious effects on either rRNA processing or cell growth. Previously, our lab has shown that the parent plasmid pFL20/5.8i4 is neutral in all aspects. That is, the 5.8S rRNA was expressed efficiently to levels were the plasmid derived population accounted for 50-60% when rRNA was isolated form ribosomes. Subsequent analyses indicated that the cells has normal growth rates and polyribosomal profiles. Further to this as constant distribution of tagged 5.8S rRNA in monoribosomes as well as small and large polyribosomes was reported (Abou Elela et al., 1995). In an earlier study, when a different tag was introduced into the 5.8S rRNA gene the growth rate of the cells was increased and this was shown to be due to a translocation defect during translation as witnessed by an increase in polyribosomes (Abou Elela et al., 1994). As can be seen in table 2.1, the newly constructed vector pFL20/18Pst5.8i4 has equal: expression levels of 5.8S rRNA, a similar copy number and a virtually identical growth rate to the parent plasmid. We take this to mean that the Pst 1 "tag" introduced into the first variable region is also functionally neutral as the expression characteristics are similar to the known neutral pFL20/5.8i4. This new construct not only satisfies the criteria of allowing us to study signals in the 5' ETS and subsequent 18S rRNA maturation, but by virtue of have

neutral "tags" in RNAs from both subunits, allowed us to examine whether an interdependent relationship exists between the RNAs of the two subunits during processing.

Chapter 3. *In vivo* analysis of large deletions in the 5'ETS rDNA from *S. pombe.*

A. Introduction

The rRNA processing event/s that are best characterized involve removal of the 5' ETS sequences. As detailed in chapter one, many trans-acting factors have been identified that are essential for these events; those include: snoRNP's, exonucleases, endonucleases, helicases, ribosomal and non-ribosomal proteins. In all cases, depletion or deletion of these factors causes an inhibition of the cleavage events A0, A1 and A2 (Fig. 1.2) The identification of multiple trans-acting factors shown to be essential for 5' ETS processing coupled with the terminal balls seen at the 5' end of nascent rRNA transcripts during electron microscopy studies, has led to the hypothesis that this spacer is removed by a large "processome" complex potentially consisting of all of the trans-acting factors (Mougey, 1993b).

Initially, the characterization of cis-acting signals was limited to *in vitro* work, but recently, systems have been developed to study these signals *in vivo* in the yeast *S. cerevisiae*. The *S. cerevisiae* 700 nucleotide 5' ETS folds into ten helical domains (I-X) and five single stranded regions (Yeh and Lee, 1992). There was an abolishment of 18S rRNA production when five large deletion mutants that spanned this spacer were constructed and assayed by northern blot analysis (Musters et al., 1989). Unfortunately, all the deletions had at least 60 bp in common so conclusions regarding different structural features of this spacer can not be drawn. This study however, did reveal the importance of the sequences within the 5' ETS for 18S rRNA maturation. Subsequently, finer mutational analyses revealed that the integrity of the first and third helical domains are not important (Lindahl et al., 1994, van

Nues et al., 1995). But when the second extended helix was removed, no mature 18S rRNA was produced which demonstrates this helix's importance for 18S rRNA maturation (van Nues et al., 1995).

The most highly studied cis-acting signal is the binding site for the trans-acting U3 snoRNP. U3 snoRNA has been cross-linked to the 5' ETS of the pre-rRNA in : mammalian, (Kass et al., 1987) *S.cerevisiae* (Beltrame and Tollervey, 1992) and *Xenopus* (Mougey et al., 1993). In *S. cerevisiae*, the cross-linked site was at a position of 230 nucleotides upstream of the mature 5' end of 18S rRNA which was proposed to be a single stranded region. Ten nucleotides of the 5' ETS at this site have the potential to form ten perfect base pairs with a portion of the U3 snoRNA. Substitution of these sequences was shown to abolish processing at the A0, A1 and A2 cleavage sites (Beltrame et al., 1994) and when compensatory changes were introduced into the U3 snoRNA molecule processing was restored (Beltrame and Tollervey, 1995). In all studies, either on trans-acting factors affecting 18S rRNA maturation or cis-acting signals of the 5' ETS, reported there was no reported effect on the maturation of the LSU rRNAs.

This chapter reports a mutational survey of the 5' ETS of *S. pombe* in order to delineate the essential features present in this spacer. Seven deletion mutants were generated by PCR-based deletion mutagenesis that span the entire 1312 nucleotides of the *S. pombe* 5'ETS. These mutants selectively delete individual or multiple helices. Following mutagenesis, the *in vivo* expression of both the SSU rRNA as well as the 5.8S rRNA from the large subunit of constructs carrying the mutagenized spacers was determined using the system described in chapter two. The results show that except for the first and last helices,

the spacer contains structural features which are essential for 18S rRNA maturation. Our model of an interdependency in processing of the SSU and LSU rRNAs is also supported by this data as deletions that affected the production of 18S rRNA also affected the production of the 5.8S rRNA molecule. As part of this work, two new PCR-based mutagenesis strategies were developed which allowed for deletion mutations to be constructed in regions that are highly repetitive.

B. Materials and Methods

The majority of procedures used in these studies have been detailed in chapter two. Alterations to those described, or new methods utilized are given below.

1. Structural predictions of the 5' ETS of *S. pombe*

The structure of the 5' ETS of *S. pombe* was predicted (Fig 3.1), using updated computer analyses with the mfold web server, (http://www.ibc.wustl.edu/~zuker/rna/form1.gci), which is based on the algorithms of Zuker and coworkers (Walter et al., 1994).The *S. pombe* sequence was previously reported by Good et al., (1997b). The folding was performed at a temperature of 30°C.

2. PCR-based mutagenesis and *in vivo* expression of mutated 5' ETS sequences.

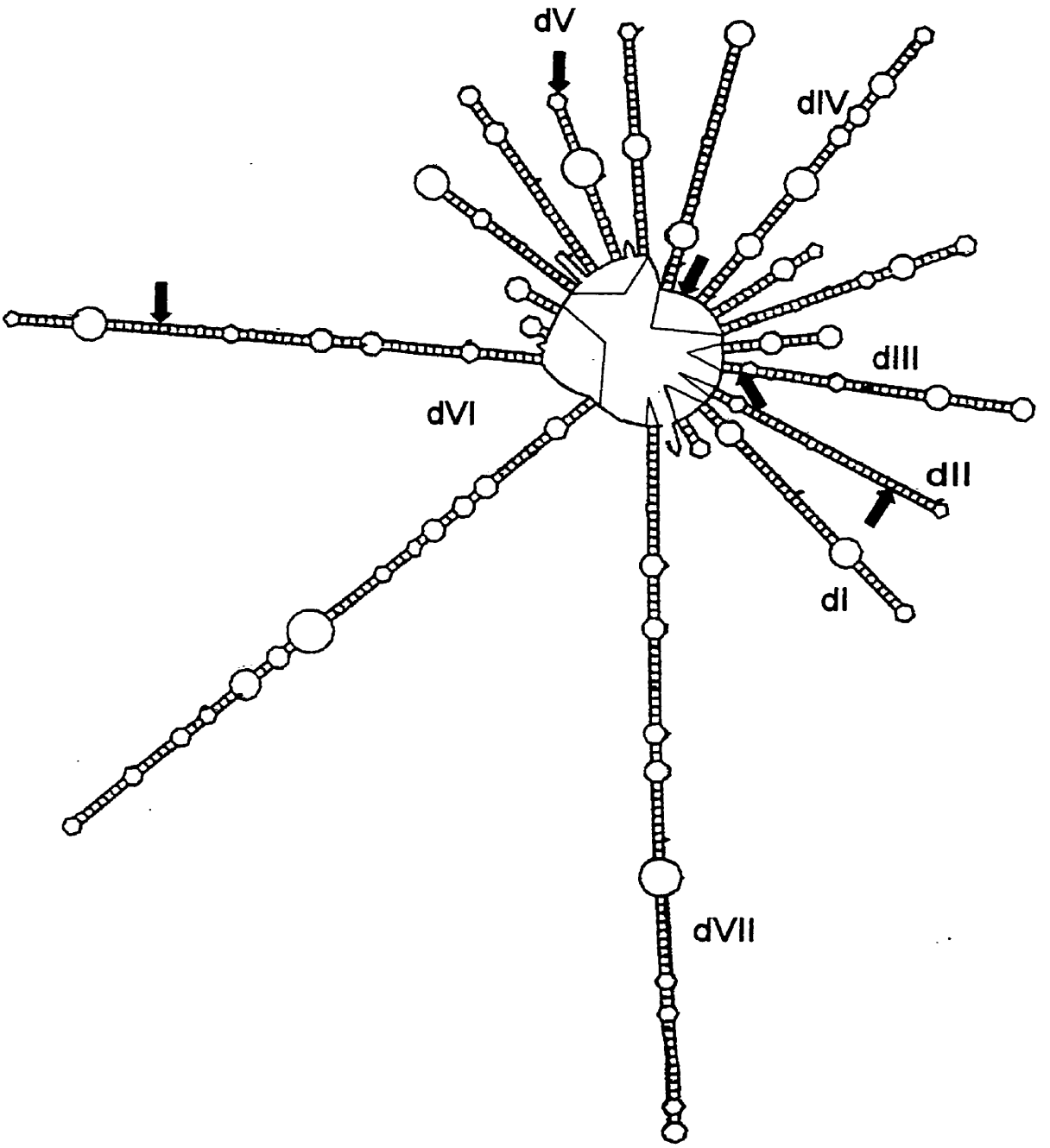
In the course of this study, two different methods were developed for introducing mutations into the 5' ETS of *S. pombe*. As such, the PCR-based mutagenesis techniques used to introduced mutations in this chapter are described in the results section.

3. Primer extension analysis

The termini of mature and precursor ribosomal RNA transcripts were detected by primer extension using reverse transcription (McKnight and Kingsbury, 1982). The 18S rRNA- tag specific oligonucleotide primer was labelled at the 5' end using [γ-^{32}P] ATP and polynucleotide kinase (Chaconas and van der Sande, 1980). Total cell RNA (10µg) was mixed with 25 ng labelled primer in 10µl 34 mM Tris/HCl, pH 8.3, 50 mM $MgCl_2$ and 5 mM dithiothreitol. Unlabeled dNTPs (dCTP, dATP, dGTP and TTP) were added to 50µM each and the mixture was incubated for one minute at 65°C before slowly being cooled to 37°C over five minutes. Moloney murine leukemia virus (MMLV) reverse transcriptase was

Figure 3.1. Estimate for the secondary structure of the 5'ETS sequence in *S. pombe* 35S pre-rRNA.

The sequence for the 5' ETS region of *S. pombe* precursor rRNA has been previously reported (Good et al., 1997b). This sequence was submitted to the mfold web server (http://www.ibe.wustl.edu/~zuker/rna/forml.cgi). The sequence was submitted to be folded at 30°C and a number of secondary structure models were predicted. The processing sites defined by Good et al., (1997b) are indicated with the bold arrows. Seven deletion mutations were created in this study to delineate essential structural features of the 5' ETS of *S. pombe*. The bases that were removed in each of these deletions are demarcated by the lines. The mutations have been labelled dI - dVII starting at the beginning of the 18S rRNA sequence and moving towards the 5' end of the transcript.


dV
dIV
dIII
dII
dI
dVI
dVII

added (2- 4 units), the reaction was incubated for 30 minutes at 37°C and terminated on ice with the addition of 15 µl sequencing stop solution. The extended cDNA fragments were characterized by electrophoresis in 6% (w/v) polyacrylamide sequencing gels, with standard dideoxy sequencing reaction products included as fragment length markers.

4. Identification of yeast transformants

Yeast transformants carrying deletions were confirmed by PCR amplification. A sterile pipettor tip was touched to a yeast colony growing on a selective plate and added to a PCR reaction mixture containing an oligonucleotide primer pair that flanked the deletion, in a method similar to that used for bacterial colony PCR identification (Gussow and Clackson, 1989). The PCR reaction consisted of an initial denaturation step with ten minutes at 95°C, 95°C one minute, 40°C one minute, 72°C for one and a half minutes; followed by thirty cycles of amplification consisting of : 95°C one minute, 50°C one minute, 72°C for one and a half minutes. The reaction products were fractionated by agarose gel electrophoresis and fragments with deletions were identified by their anticipated size.

C. Results

1. Structure of the 5' ETS in *S. pombe* rRNA.

The secondary structure of the *S. pombe* 5' ETS was predicted using the mfold web server (http://www.ibc.wustl.edu/~zuker/rna/form1.gci) which is based on the algorithms of Zuker and coworkers (Walter et al., 1994). The previously determined 5' ETS sequence (Good et al., 1997b) was submitted and estimates of similar stability were returned .The estimate that was predicted to be the most stable (ie highest change in free energy) was chosen to be the working model. As can be seen in Figure 3.1, the structure is highly paired folding into many extended helices that are separated by relatively short single stranded regions.

2. Development of novel PCR-mutagenesis strategies that allowed for the creation of large deletions in the 5' ETS sequences.

The incorporation of mutagenic oligomers during DNA amplification by the PCR has become a widely used method for introducing site-specific mutations into target DNA (Newton and Graham, 1994, Silver et al., 1995). However, during the course of this work it was found that DNA segments, which contain repeated elements, may remain very difficult to mutate due to *in vitro* recombination events which can result in very heterogeneous populations of products. For example, using standard protocols such as the "mega-primer" method (Sarkar and Sommer, 1990), attempts to introduce deletion mutations (50-100 bp.) into the repetitive regions in the 5' ETS of *S. pombe* ribosomal DNA , always resulted in broad streaks (Fig. 3.2A). All standard modifications to increase the specificity of the reaction by alterations in annealing temperature (37°C-65°C, five degree

increments) annealing times (one minute-15 minutes, two minutes increments), amount of primer (10-100 ng), amount of template (1-50 ng), magnesium chloride concentration (0.25 mM-3 mM, 0.25 increments) and cycle number (20-35) provided little improvement. "Hot start" PCR (D'Aquila et al., 1991) as well as "Touchdown" PCR (Don et al., 1991), methods which have been designed to improve the specificity of the reaction, also gave the same unsatisfactory results (data not shown). The "overlap extension" method (Ho et al., 1989) which like the "mega-primer" method was designed initially to embed mutations within a DNA sequence by PCR amplification was also attempted. This method has been shown to be a more efficient technique for generating large deletion mutations (>200 bp.) (Senanayake and Brian, 1995). However, as found with the "mega-primer" method, the first PCR amplifications of the overlapping fragments were successful, but the extension step again yielded an extremely heterogeneous mixture of products (Fig. 3.2B). Apparently, alternate pairing between the complementary strands continued to provide heterogeneous initiations for DNA replication which resulted in highly recombined populations of replicated molecules.

Because it was believed that the observed product heterogeneity was likely the result of recombination between complementary sequences in the opposite strand, these competing factors were removed using an overlap extension strategy with only one set of complementary single strands (Fig. 3.3A). Using methods previously developed for DNA sequencing by chemical degradation (Maxam and Gilbert, 1980), the products of the first PCR amplifications were strand separated and gel purified (Fig. 3.3B).The isolated complementary strands were then annealed, extended by Taq polymerase and finally this

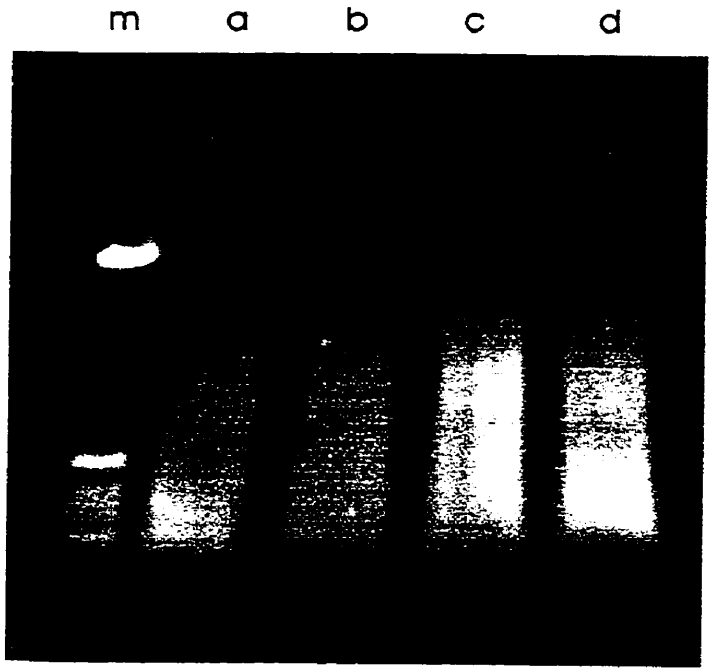
template was PCR amplified with normal primers to yield the 500 base pair desired product. As shown by the example in Fig. 3.3C, this approach was successfully applied to generate mutations (dI-dIII) (Fig. 3.1) yielding very satisfactory results with only a single major mutant product. In each case the mutations were confirmed by DNA sequencing. The added strand separation step clearly allowed for the efficient and specific amplification of the desired product providing for mutations which previously could not be made.

At the same time, an additional PCR-based mutagenesis strategy, was utilized to generate the mutations dIV-dVII (Fig. 3.1).To aid use of this technique a new template plasmid was constructed. The vector pFL20/18Pst5.8i4 was digested with Pst 1 and Hind III restriction endonucleases. A DNA fragment which contained the 5' ETS sequence and the first 70 base pairs of the 18S rRNA gene was ligated to pTZ19R and digested with the same restriction endonucleases which gave rise to the template plasmid, pSp5'ETSP (Fig. 3.4) As can be seen in Fig. 3.4, using this new template two independent PCR amplifications were conducted using the same conditions described in chapter two, section 4(a). This gave rise to the products shown in Fig. 3.4ii. In each case, one of the primers was first phosphorylated at the 5' end using T_4 polnucleotide kinase and the conditions described in chapter two, section 4(e). The products of the reactions were cut with either Hind III (left reaction) or Pst 1(right reaction) restriction endonucleases, and ligated with p5'ETS18S5.8Cla cut with the same restriction endonucleases. The mutated sequences were then used to replace the normal sequences in pFL20/18Pst5.8i4 and their *in vivo* expression was determined.

Figure 3.2. Attempts at a PCR-mediated site specific deletion in the 5' ETS of *S. pombe* rDNA.

(A) Heterogeneous DNA amplification during PCR-mediated mutagenesis using the "mega-primer" technique (Sarkar and Sommer, 1991). DNA was amplified by PCR in 50 µl of 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.5, containing 0.1% Triton X-100, 200 µM of each dNTP, 100 µg/ml BSA, 50 ng of each primer, 50 ng of template DNA and one unit of Taq DNA polymerase. An initial denaturation step at 95°C for five minutes was followed by 33 cycles of denaturation at 95°C for one minute, annealing at different temperatures for one minute and elongation at 72°C for 45 seconds. The PCR products after fractionation on a 1% agarose gel, with annealing at 40°C annealing temperature (a), 45°C (b), 50°C (c) and 55 °C (d), are shown together with Hinf I digested pBR322 as a size marker (m). (B) Heterogeneous DNA amplification during PCR mediated mutagenesis by the "overlap extension" method (Ho et al., 1989). Products of the second reaction using a 50°C annealing temperature and the same PCR cycle conditions (a) were fractionated on a 1% agarose gel and stained with ethidium bromide.

A.



B.

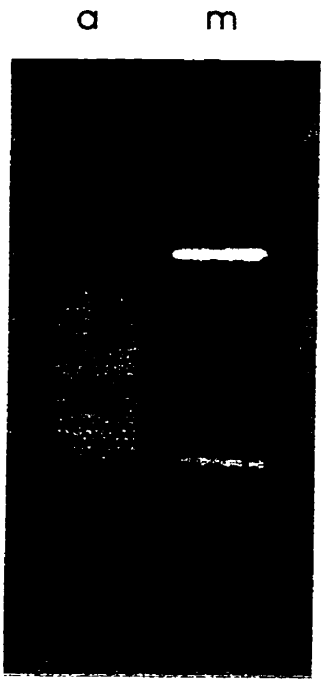
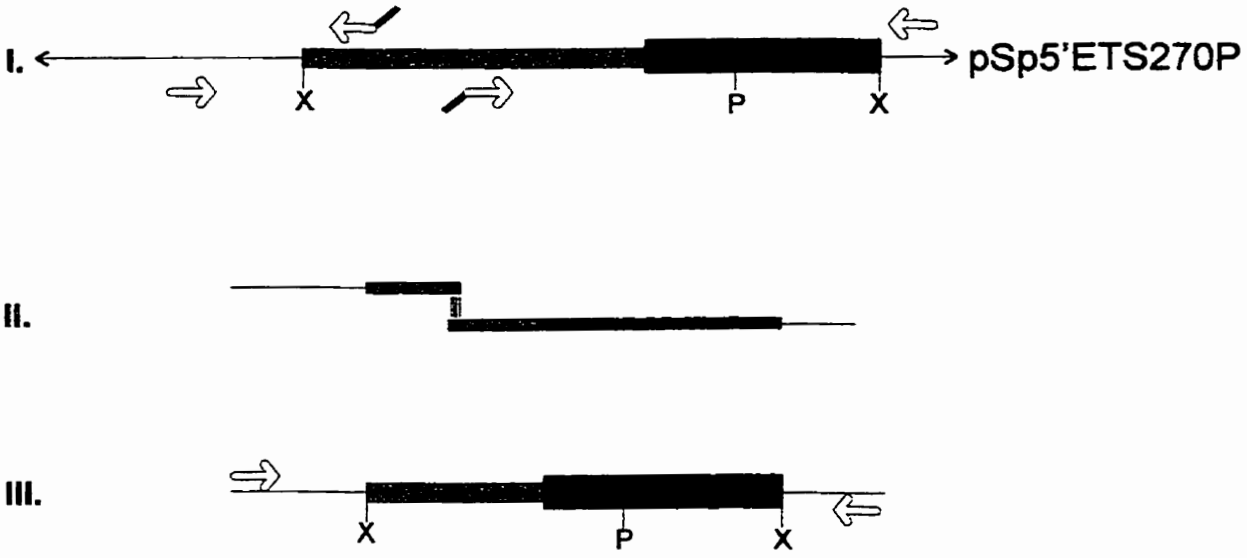


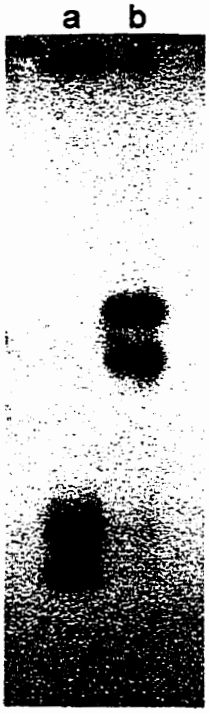
Figure 3.3. Homogeneous DNA amplification using PCR-mediated mutagenesis by single strand overlap extension (SSOE).

A.(I) Initially, two independent PCR amplifications were performed utilizing normal primers (open arrows) and primers that contained 15 bases of complementarity at their 5' Ends (black extensions on open arrows) (Bi). (II) The products from step I are strand separated, fractionated by gel electrophoresis and eluted by gel homogenization before the complementary strands are annealed and extended at 72°C for five minutes using Taq DNA polymerase. To form the complementary hybrid, one strand from the first reaction is hybridized separately with each strand from the other reaction overnight at 37°C in 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCL, pH 7.5, containing 0.1% Triton X-100. (III) The template generated in step II is PCR amplified. Since complementary strands are not identified in advance, both hybrid reactions are amplified with only one resulting in product of the desired size. Normal and mutagenic primers are indicated by open and closed arrows, respectively. All PCR reactions were performed using a 50°C annealing temperature; the primers and cycle conditions were those described in Fig. 3.2. When autoradiography was used to detect the products, 5 μCi of [$\propto$-^{32}P]dCTP were included in the reaction mixture. (B) Autoradiograph of strand separated products produced during step I. Double stranded PCR products using primers 1 and 2 or 3 and 4 were denatured in 30% DMSO (v/v) and separated on an 8% non-denaturing polyacrylamide gel overnight at 400 V at 4°C. (C) Site directed deletion of a 52 base pair hairpin structure in the 5' ETS of the *S. pombe* rDNA. Products of step III were fractionated on a 1% agarose gel and stained with ethidium bromide (a). A Hinf I digest of pBR322 is included as a size marker (m).

A



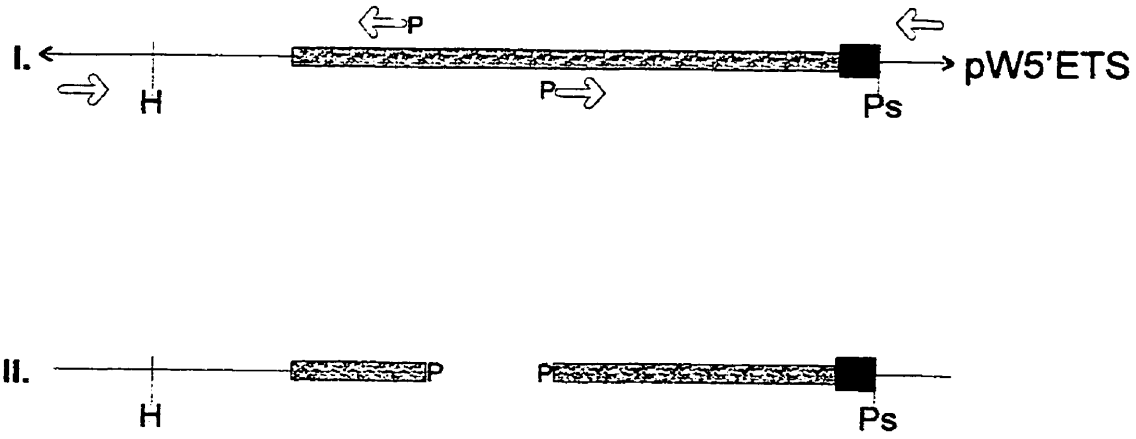
B



C

Figure 3.4. PCR-based mutagenesis strategy used to delete specific regions of the 5' ETS rRNA sequences in *S. pombe*.

A. Deletions created by performing two independent reactions. In the first step AI, two independent PCR amplifications were performed using non-mutagenic oligonucleotides. In each case, one of the primers had a phosphate group added to the 5' end (indicated by P) (AI). The products of the two reactions were then gel purified and cleaved with either Hind III (H) or Pst 1(Ps) restriction endonucleases. The products were then ligated with the vector pSp5'ETSP cut with the same restriction endonucleases (AII).

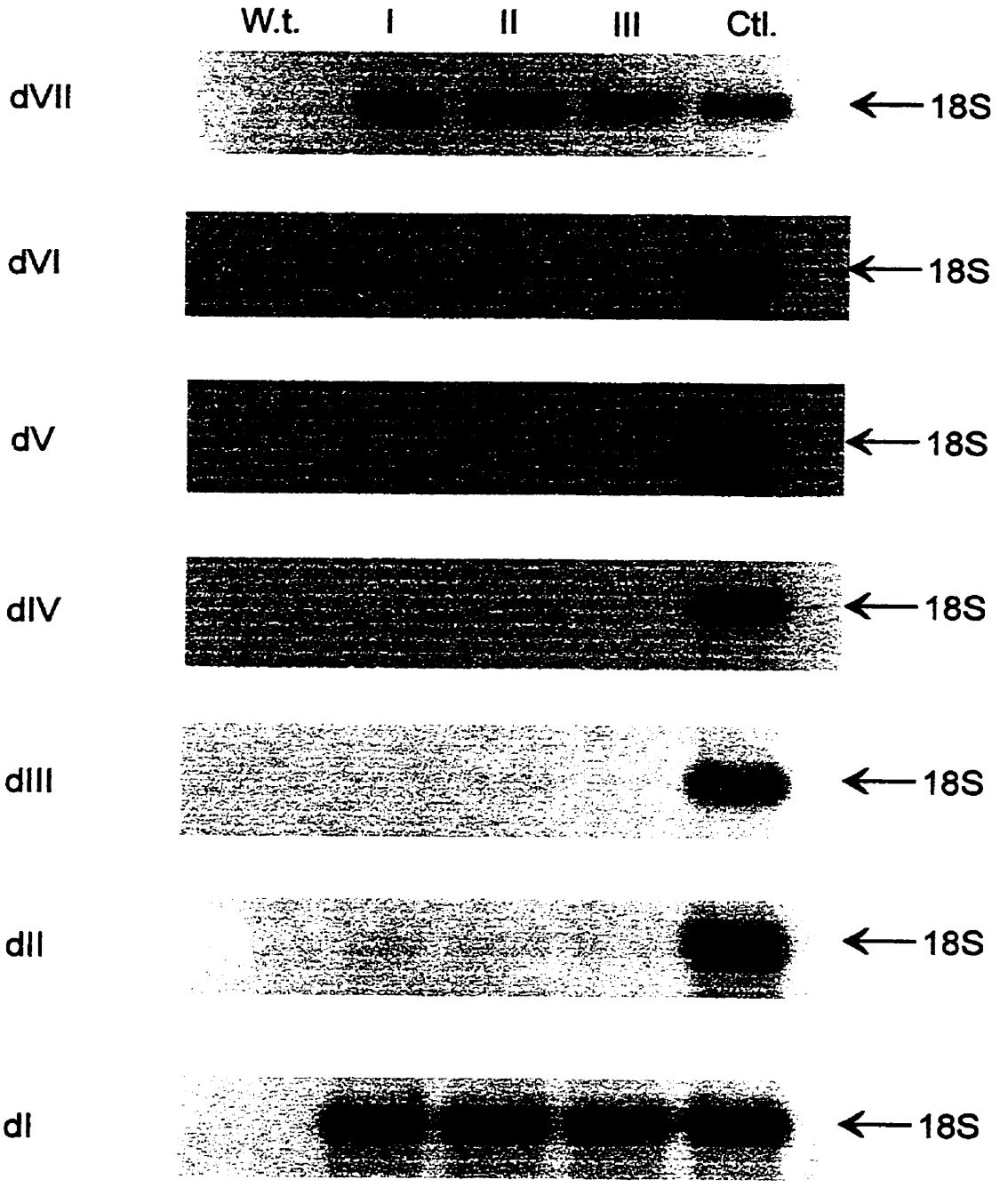

I.
P
H
P
Ps
pW5'ETS
II.
H
P
P
Ps

2. Effect of 5'ETS sequence deletions on rRNA processing and ribosome biogenesis in *S. pombe*.

To examine the importance of the predicted structural features in rRNA maturation, seven non-overlapping deletion mutants were systematically introduced into the 5'ETS structure using either the PEP (dIV-dVII) or the SSOE (dI-dIII) PCR-mediated mutagenesis strategies. As can be seen in Fig. 3.1, the mutations sequentially removed sections of the structure beginning at the 5' end of the rRNA precursor molecule. Each mutant rDNA was used to transform *S. pombe* cells to permit its' expression *in vivo* and, as previously described in chapter two, the neutral marker in the 18S rRNAs was used to study the maturation of the pre-rRNA transcript and to measure the amount of mature 18S rRNA which was derived from the mutant genes. As shown in Figure 3.5 , when the marker-specific probe was used for hybridization analyses, all normal plasmid controls (Ctl) indicated substantial amounts of mature plasmid-derived 18S rRNA and no tagged RNA was detected in extracts of untransformed cells (Wt). The amount of tagged RNA, however, varied with the seven deletion mutants. When the 5' most helix was deleted (dVII), there was no detectable difference in 18S rRNA production between the control and mutated sequences and deletion of the helix (dI), most proximal to the mature 18S rRNA, resulted in only modestly lower (83.0±4.2% of the control level) levels. But, when the deletion mutants dII - dVI were assayed, in each case, no mature 18S rRNA was detected. To ensure a reproducible effect, the analyses were performed with three individual transformants for each mutant type and in each case, the membrane was stained with methylene blue as described in chapter two (Fig. 2.5) in order to ensure that comparable amounts of RNA were

Figure 3.5. Expression of plasmid-derived 18S rRNA in *S. pombe* cells containing deletions in the 5'ETS sequence.

Whole cell RNA was prepared from *S. pombe* cells transformed with mutant rRNA genes and fractionated on agarose/formaldehyde gels (Good et al., 1994; 1997a). RNA from untransformed *S. pombe* cells (Wt) and cells transformed with normal but "tagged" (Ctl) *S. pombe* rDNA (Good et al., 1997a) were included on each gel. Gels were blotted on nylon and hybridized (Rose et al., 1990) with a ^{32}P-labelled oligomer, specific for the tagged 18S rRNA transcribed from the plasmid-associated rDNA (Good et al., 1997a). As detailed in chapter two (Fig. 2.4), each membrane was stained with methylene blue prior to hybridization to confirm that equal amounts of RNA were transferred or to normalize the hybridization results when there was unequal loading of the sample. The results presented show three individual transformants for each deletion to ensure a reproducible effect was observed. The position of the mature 18S rRNA is indicated on the right. The quantification results for these studies are summarized in table 3.1


W.t.
I
II
III
Ctl.
dVII
dVI
dV
dIV
dIII
dII
dI
18S
18S
18S
18S
18S
18S
18S

analysed or to normalize the hybridization results for any loading variations.

In light of the effects on 18S rRNA which were observed with deleted regions II - VI, the effects of these deletions on the maturation of the LSU RNAs also were examined. As previously described in chapter two and illustrated in Figure 3.6, the maturation of plasmid-derived 5.8S rRNA could be quantified accurately through the use of a neutral four base insertion which resulted in a distinct electrophoretic mobility (labelled plasmid-derived RNA in Fig. 3.6). Under the growth conditions which were used in this study, approximately 52% of the mature 5.8S rRNA is plasmid-derived. As also shown in Figure 3.6 and summarized in table 3.1, the deletion of the first hairpin (dVII) also had no effect on LSU rRNA production and only a moderate effect was seen when dI was assayed with approximately 80% of the normal amount being evident. The levels with dII-dIV deletions, however, were substantially lower at about 40% of normal and, most strikingly, the levels produced with dV and dVI were below detectable levels.

Since some mutations had severe effects on the production of 18S rRNA, the precursor levels in these cases also were determined to evaluate changes in the RNA precursors. As can be seen in Figure 3.7, two different phenotypic classes were observed. In the case, of deletions dIV-dVI, where the level of 5.8S rRNA was approximately 40% of the control, no primary transcript could be detected. Instead, a novel band that migrated just below the mature 25S rRNA was evident (Fig. 3.7B). Whereas when the precursor levels for dII and dIII were assayed, a significant build-up of the primary transcript clearly was evident (Fig 3.7A).

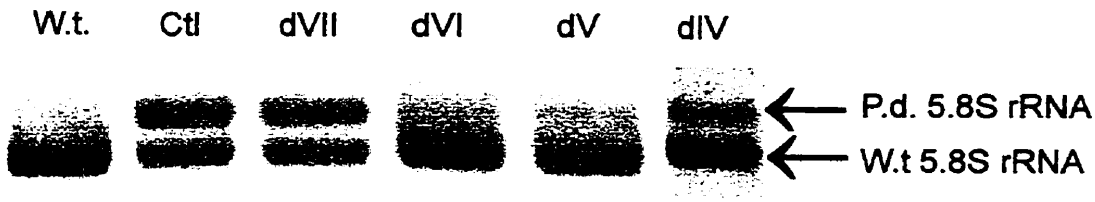
Since 3' most helix (dI) did have some effect on the level of mature 18S rRNA

Figure 3.6. Expression of plasmid-derived 5.8S rRNA in *S. pombe* cells containing deletions in the 5'ETS sequence.

A. Whole cell RNA was prepared from *S. pombe* cells transformed with mutant rRNA genes containing deletions (VII-IV), fractionated on a polyacrylamide gel (Abou Elela et al., 1995) and stained with methylene blue. RNA from untransformed *S. pombe* cells (Wt) and cells transformed with normal but "tagged" (Ctl) *S. pombe* rDNA (Good et al., 1997a). The positions of the normal (Wt. 5.8S rRNA) and tagged (plasmid-derived) 5.8S rRNAs are indicated on the right.

B. Whole cell RNA was prepared from *S. pombe* cells transformed with mutant rRNA genes containing deletions (I-III), fractionated on a polyacrylamide gel (Abou Elela et al., 1995) and stained with methylene blue. RNA from untransformed *S. pombe* cells (Wt) and cells transformed with normal but "tagged" (Ctl) *S. pombe* rDNA (Good et al., 1997a). The positions of the normal (Wt. 5.8S rRNA) and tagged (plasmid-derived) 5.8S rRNAs are indicated on the right.

A.


W.t.
Ctl
dVII
dVI
dV
dIV
P.d. 5.8S rRNA
W.t 5.8S rRNA

B.

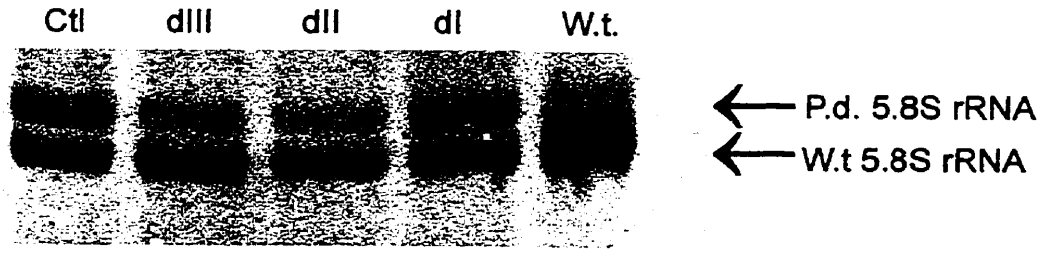
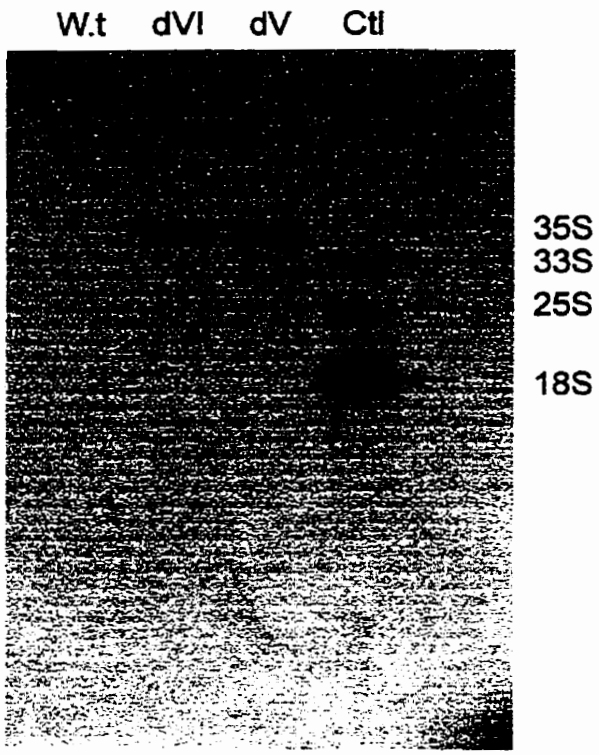

Ctl
dIII
dII
dI
W.t.
P.d. 5.8S rRNA
W.t 5.8S rRNA

Figure 3.7. Precursor analysis of mutations that were shown to be essential for 18S rRNA production.

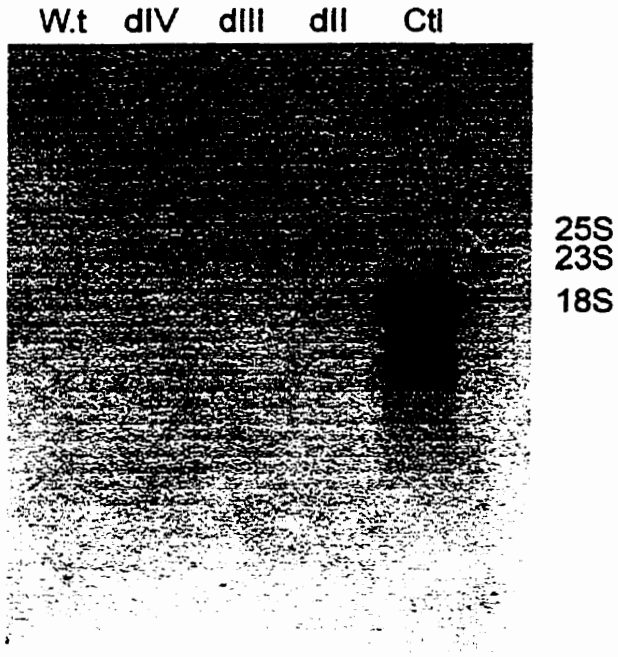
Whole cell RNA was prepared from *S. pombe* cells transformed with mutant rRNA genes and fractionated on agarose/formaldehyde gels (Good et al., 1994; 1997a). RNA from untransformed *S. pombe* cells (Wt) and cells transformed with normal but "tagged" (Ctl) *S. pombe* rDNA (Good et al., 1997a) were included on each gel. Gels were blotted on nylon and hybridized (Rose et al., 1990) with a ^{32}P-labelled oligomer, specific for the tagged 18S rRNA transcribed from the plasmid-associated rDNA (Good et al., 1997a). Each membrane was stained with methylene blue (see chapter two Figure 2.5) prior to hybridization to confirm that equal amounts of RNA were transferred or to normalize the hybridization results for unequal loading. A. The origin of the RNA that was blotted is indicated at the top. The 18S and 25S rRNA positions as well as known precursor bands are indicated at the right.

B. The origin of the RNA that was blotted is indicated at the top and the 18S and 25S rRNA positions are indicated at the right. Also, the presence of a novel band is indicated as 23S.

A.



B.



(refer to Table 3.1) the 5'- terminal also was evaluated for evidence of aberrant processing. As shown in fig 3.8, primer extension analyses indicated a normal terminal in the 18S rRNA (site A_1) was evident in each case, but the A_0 cleavage site in the mutant precursor was evident as a shorter fragment closely corresponding with the 83 nucleotide deletion. The deletion of the helix most proximal to the mature 18S rRNA, therefore, does not influence the position of cleavage either at A_0 or A_1 although it does appear to reduce the yield of the mature 18S rRNA.

Finally, in order to ensure that the differences in 18S rRNA yield were not the result of effects on plasmid replication, the relative plasmid copy number and growth rates were examined in each transformant as described in chapter two and the results are summarized in table 3.1. As can be seen the introduction of mutations in the 5' ETS did not have effects on either the copy number of the plasmid or the doubling time of transformant colonies which carried plasmids with these mutations.

Figure 3.8. Primer-extension analysis of termini in the 5'ETS region of *S. pombe* rDNA mutants most proximal to the 18S rRNA.

Whole cell RNA was prepared from *S. pombe* cells transformed with "tagged" rDNA (Ctl) and cells transformed with "tagged" rDNA containing a deletion in the first (I), second (II) or third (III) hairpin region, and hybridized with an oligonucleotide primer (GTCAACCTGCAGTATAC) specific for the 5'-end region in the plasmid encoded 18S rRNA (Good et al., 1997a). The primer was extended with Moloney murine leukemia reverse transcriptase and the products were fractionated by polyacrylamide gel electrophoresis. Standard sequencing reaction products (G, A, T and C) were also applied as nucleotide markers. The positions of termini for the mature 18S rRNA and two precursor intermediates (A_o and $A_{o'}$), as previously reported by Good and coworkers (1997b), are indicated on their right.

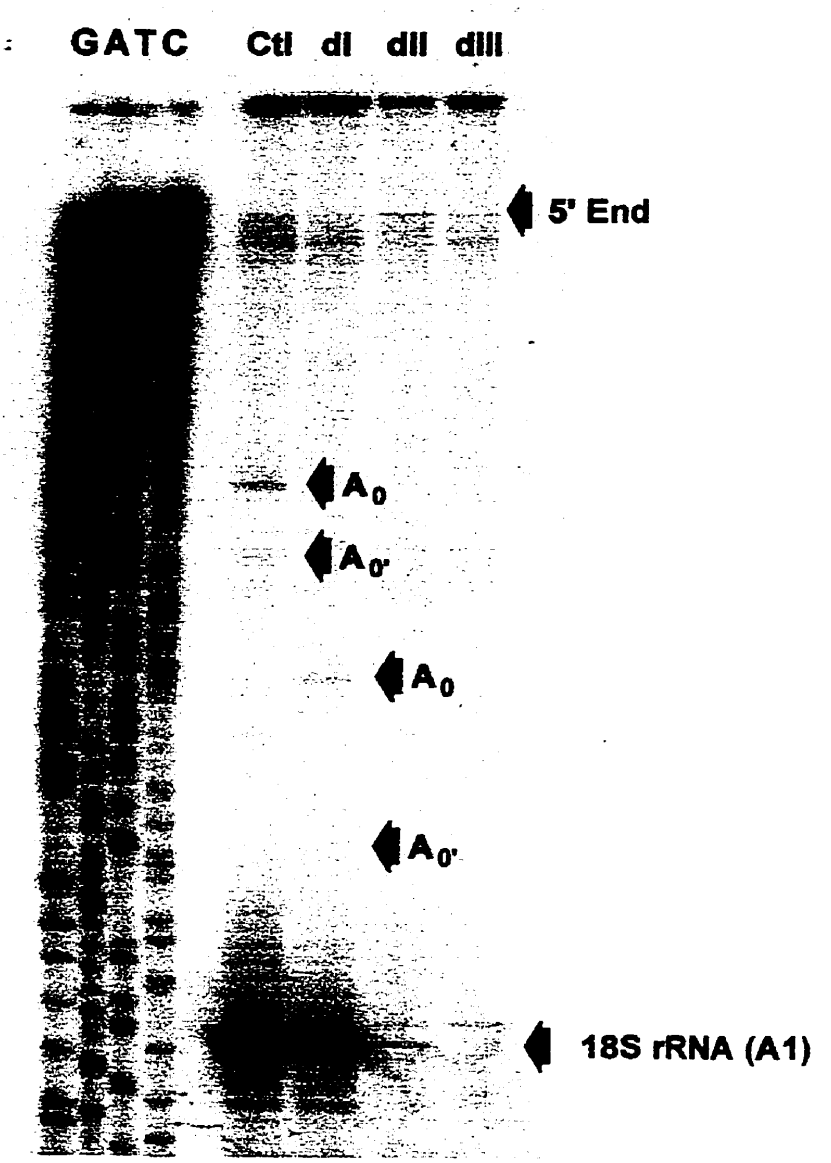

GATC
Ctl
dI
dII
dIII
5' End
A0
A0'
A0
A0'
18S rRNA (A1)

Table 3.1 Growth characteristics of *S. pombe* cells expressing mutations in the 5' E TS.

Source of RNA	Plasmid derived 5.8S rRNA[a]	Plasmid derived 18S rRNA[b]	Relative Plasmid Copy number[c]	Doubling Time of Transformants[d]
Control	52.3 +/- 1.1	100	74 +/- 1.2	4.3 +/- 0.1
dVII	51.8 +/- 2.4	104	74 +/- 2.3	4.3 +/- 0.1
dVI	trace	0	72 +/- 1.1	4.2 +/- 0.1
dV	trace	0	73 +/- 2.6	4.3 +/- 0.3
dIV	18.4 +/- 3.2	0	74 +/- 1.0	4.3 +/- 0.1
dIII	20.8 +/- 1.1	0	73 +/- 1.0	4.3 +/- 0.1
dII	21.6 +/- 1.0	0	76 +/- 0.6	4.3 +/- 0.1
dI	39.1 +/- 1.1	82.3 +/- 3.5	73 +/- 0.96	4.3 +/- 0.1

a. Mean values are expressed as the percentage of plasmid-derived 5.8S rRNA. Values are calculated from at least four replicate experiments. The value of the standard deviation is given.

b. Mean values are expressed as a percentage relative to control (100%) and are calculated from at least four replicate experiments. The value of the standard deviation is given.

c. Copy numbers are expressed relative to the control plasmid and are calculated from at least three replicate experiments. The mean value and the standard deviation is given.

d. Doubling times in hours were determined as described in table 2.1 and the reported values are from three replicate experiments. The mean value and the standard deviation is given.

D. Discussion

Removal of the 5' ETS sequence in the pre-rRNA molecule has been the most studied event in rRNA maturation. Studies *in vitro* and *in vivo* have shown that numerous trans-acting factors are essential for these processing events to proceed. However, the discovery of essential structural elements has not been as fruitful. This, however, may be due in part, to the fact that only recently have appropriate systems been developed that allow such evaluations. Even with the development of these systems most of the focus has been placed on defining the sequence within this spacer that interacts with the U3 snoRNP complex. In the present study, an efficiently expressed *in vivo* mutational analysis system was used to perform a mutagenic survey of the 5' ETS of *S. pombe* in order to further delineate any essential sequence or structural features in the whole spacer.

Initially, the 5' ETS sequence of *S. pombe* was submitted for structural analysis to the mfold web server (http://www.ibe.wustl.edu/~zuker/rna/form1.cgi). As can be seen in Figure 3.1, the RNA sequences are predicted to fold into a highly paired structure with very few single stranded regions which is consistent with structures predicted for 5' ETS sequences of other organisms.

Based upon the structural estimate, seven consecutive deletion mutants were generated that spanned the entire spacer and the results presented here show that the expression of the 18S rRNA in these mutants was variable. Deletion of the first helix (dVII), or the last helix (dI), had no or moderate effect on the production of 18S rRNA which is consistent with what has previously been reported. Lindahl and co-workers (1994) documented that deletion of the first helix did not effect the maturation of 18S rRNA, and

in another study van Nues and co-workers, (1995) showed that removal of the top portion of the helix closest to the mature 18S rRNA again had no effect on production of the SSU rRNA. The fact that in the current study a modest effect with the dI mutation was observed may be simply reflective of the fact that the whole helix was removed versus just the most apical bases in the previous work. During the course of this thesis it was reported that the formation of the proper 5' terminus of the 18S rRNA is dependent on a spacing mechanism and the actual sequences within the vicinity of the A1 site were not important (Venema et al., 1995).Since deletion of helix I did have a moderate effect on 18S production, primer extension analysis was performed to detect signs of aberrant processing and the results indicate that although the amount of 18S rRNA was reduced some what, processing of tagged 18S rRNA appears to be normal at the A1 site.

Mutations dII - dVI on the other hand had dramatic effects and in each case no tagged 18S rRNA could be detected. There are two possibilities which could account for these dramatic effects. Good et al., (1997b) defined the putative processing sites in the 5' ETS of *S. pombe* by primer extension and S1 analysis and each of the above mutations would eliminate one of these sites. Therefore, if each of these putative processing sites are essential for 18S rRNA maturation, then their removal would be expected to have negative effects on 18S rRNA maturation. This is consistent with studies on ITS1 and ITS2 in *S. cerevisiae*, where it has been shown that deletion of structural features that include processing sites have deleterious effects on mature LSU rRNA production (Allmang et al., 1996 a,b, van Nues et al., 1995b). A second possibility is that deletion of the sequences in the mutants dII-dVI in someway rendered the primary transcript susceptible to nuclease

attack and highly unstable. Two scenarios could be envisioned in this case. In the first scenario, deletion of the sequences prevents proper folding of the precursor molecule which in turn exposes nuclease sensitive sites. In the other scenario, the deleted sequences may have removed binding sites for a trans-acting factor, or perhaps a factor with multiple binding sites on the pre-rRNA molecule, such as nucleolin, which normally may protect the primary transcript from nuclease attack.

In an attempt to detect whether the abolishment of tagged 18S rRNA production was due to defects in processing or the creation of nuclease sensitive sites, the levels of the primary transcript in the mutations dII - dVI were assayed. Two very different phenotypes were seen. In the deletions that are most proximal to the 18S rRNA, (dII-dIV) no full length precursor could be detected. Instead a novel band (termed 23S) appeared. On the other hand, in the two most distal mutations (dV and dVI), a significant build-up of the pre-rRNA clearly was evident. Both classes suggest that the precursor molecule does not become sensitive to nuclease attack. The appearance of a novel 23S band has been reported in *S. cerevisiae* when processing in ITS2 is disrupted (van Nues et al., 1995b, Allmang et al., 1996b). In this case, the 23S was shown to consist of: the 5' ETS, the 18S rRNA and some ITS1 sequences. Although the novel 23S band has not been fully characterized in this study, it is known that in mutants dII - dIV the band did hybridize with the 18S rRNA oligonucleotide, an observation which suggests the presence of tagged 18S rRNA. Also, when primer extension studies were performed on mutants dII and dIII, the tag specific oligonucleotide could be extended to the 5' end of the primary transcript which suggests that this band consists of at least the 18S rRNA as well as most of the 5' ETS sequences. With the other phenotype, there

is a significant build-up of the primary transcript which clearly shows that the RNA is expressed and that the deletions have not created simply an instability in the primary transcript. Instead, an overall processing defect appears to result in a build-up of the primary transcript. Therefore, in all cases where mutation in the 5' ETS region caused an abolishment of 18S rRNA maturation, it appears that there is an inhibition of processing this spacer.

In a previous study, it was reported that removal of the ITS2 sequences can reduce the amount of mature 18S rRNA produced to approximately ten percent of normal levels. (Good et al., 1997a). This processing interdependence was further examined in the current study by assessing the level of mature plasmid derived 5.8S rRNA produced in the seven 5'ETS deletions. As with the production of 18S rRNA, the levels of 5.8S rRNA seen in the mutants varied but a strong correlation existed between the two. When the 5' most helix was deleted, no effects were seen on the levels of 5.8S rRNA or the 18S rRNA. Also, when the last helix was deleted, the levels of 18S rRNA were approximately 80% of control levels and the same can be seen for the amount of plasmid-derived 5.8S rRNA. Again, the internal five mutations had dramatically different effects. Mutations dII - dIV reduced the levels of 5.8S rRNA to about 40% of control levels and when the dV and dVI mutations were assayed, only trace amounts of plasmid derived 5.8 S rRNA can be seen. As previously reported there is a strong interdependence in the processing of the SSU and LSU rRNAs in *S. pombe* which stands in contrast to that reported for *S. cerevisiae*.

The reasons for the variations seen in both the 5.8 S RNA production and precursor levels remains unclear, however, a model can be envisioned which accounts for both. The

deletions which are in closer proximity to the mature 18S rRNA resulted in the appearance of an aberrant 23S intermediate molecule and plasmid-derived 5.8S rRNA levels of approximately 40% of control levels. This shows that in these mutants processing in ITS1 can proceed, albeit, with less efficiency. As described earlier, processing of the A0, A1 and A2 sites is thought to be performed by a large "processome" complex. Therefore, it is attractive to speculate that ,in this group of mutants, the processome complex can form and although processing at A0 and A1 is inhibited (as witnessed by the 23S intermediate) processing can proceed at the A2 site at a reduced rate. However, the mutations where there is a build-up of the primary transcript and virtually no 18S or 5.8S rRNA is produced, it could be taken to mean that the deleted sequences in some manner inhibit processome formation and thereby cause a halt to processing and a corresponding build-up in the precursor levels.

In summary, there appear to be many structural features within the 5' ETS of *S. pombe* rRNA that are essential for processing of the primary transcript as a whole. More specifically, the first and last helical domains of the 5' ETS of *S. pombe* are not essential for transcript processing and their deletion results in no or modest effects on mature rRNA levels. The results presented here, also show that the putative processing sites that were identified earlier appear to be essential for the maturation of the SSU rRNA and there is further evidence of a strong processing interdependence between the SSU and LSU rRNAs. The results also raise the possibility that the more distal portions of the 5' ETS may be essential for processome formation and that inhibition of this complex formation can stop processing of the primary transcript entirely.

Chapter 4. Structural analysis on the proximal portion of the 5' ETS in *S.pombe* pre-RNA

A. Introduction

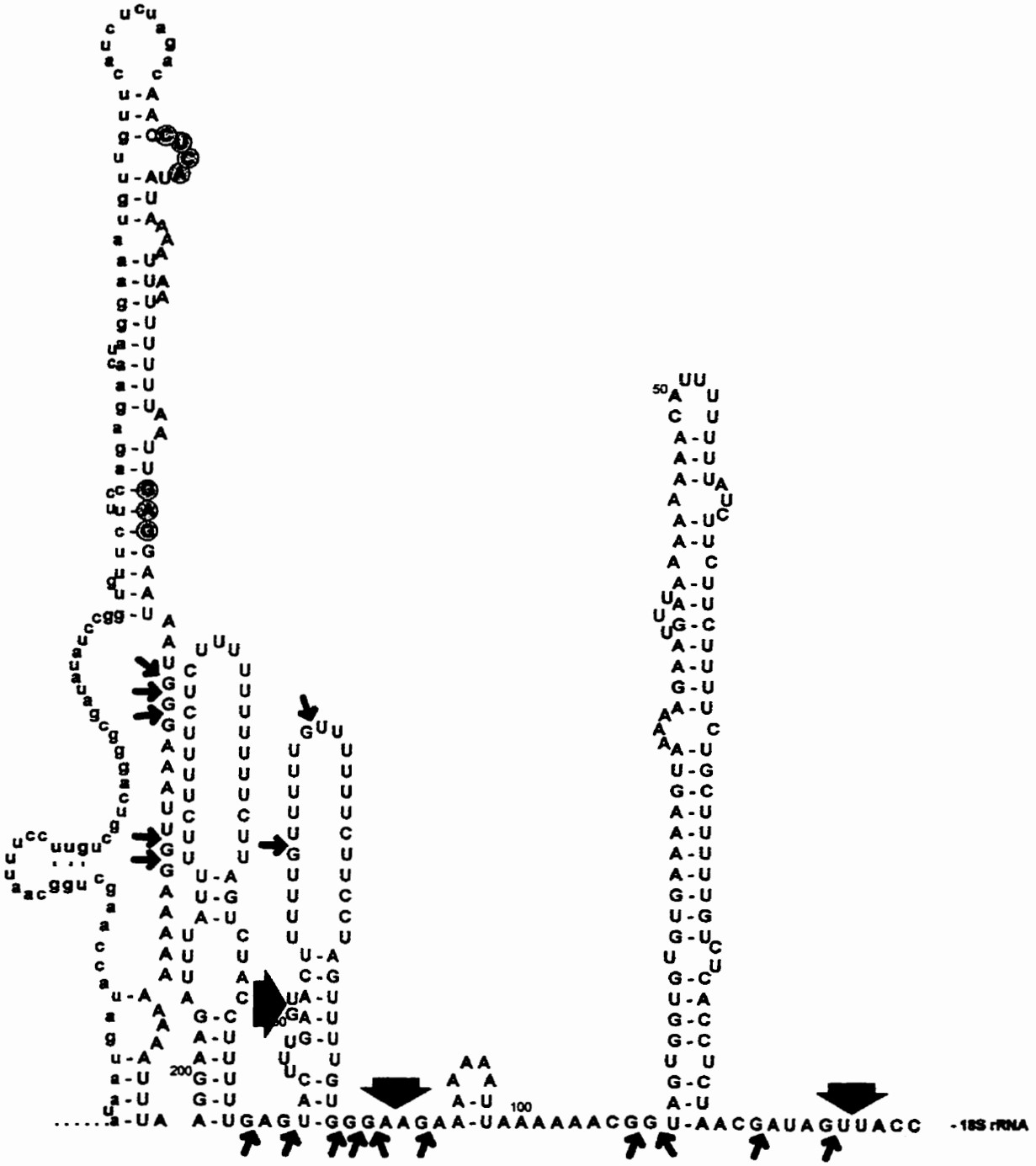
Prokaryotic pre-rRNA processing is carried out by a defined set of protein nucleases (for review see Apirion and Miczak, 1993). Key among these is RNase III, which was initially identified by its ability to bind and cleave double strand RNA (Robertson et al., 1968). RNase III releases the 16S and 23S rRNAs from the primary transcript by cleaving within two extended helical structures formed by long-range interactions that pair the termini of each rRNA (Bram et al., 1980). A RNase III homologue (RNT-1) has been identified in *S cerevisiae* which was shown to be essential for rRNA processing *in vivo* (Abou Elela et al., 1996). More specifically, depletion of this enzyme resulted in a loss of cleavage at the A0 site in the 5' ETS and the +21 site in the 3' ETS. It was shown also that purified RNT-1 could specifically cleave *in vitro* generated substrates at these sites. More recently, the RNase III homologue isolated from *S. pombe* (Pac 1) was shown to cleave *in vitro* generated substrates at two of three sites in the 3' ETS of this organism, originally identified *in vivo* by Melekhovets and co-workers (1994). Collectively, these results suggest that the structure of both the 5' and 3' ETS' are important for processing of the primary transcript.

Yeh and Lee (1992) reported a structural estimate for the *S. cerevisiae* 5' ETS based on enzymatic and chemical probing data generated from *in vitro* transcripts. In an attempt to maintain the key structural features modeled for *S. cerevisiae,* the authors proposed a structure for the *S. pombe* 5' ETS as well (Fig. 3.1). This chapter presents a reevaluation of

the 270 bases most proximal to the mature 18S rRNA based on the nucleotide sequence and its higher order structure. The results clearly indicate that the structure of the *S. pombe* 5' ETS in this area is more highly paired than previously believed and they raise new questions regarding the current structural model for yeast and the role this structure plays in 18S rRNA maturation.

Figure 4.1. A secondary structure model for the proximal region of the 5'ETS sequence in *S. pombe* 35S pre-rRNA.

The model is taken from Yeh and Lee, (1992); nucleotide residues not examined in the present study are indicated in lower case. The small arrows represent guanylic acid residues which are predicted to be readily cleaved by T_1 ribonuclease. The large arrowheads represent known cleavages during RNA maturation (Good et al., 1997b). The shaded residues are conserved in the secondary structure as described in Figure 3.2.


50
100
200
-18S rRNA

B. Materials and Methods

1. 5' ETS structural estimates of *S. pombe* and *S cerevisiae.*

The secondary structure of the 270 bases most proximal to the 18S rRNA sequences were predicted. The *S. cerevisiae* sequence submitted was taken from Yeh and Lee, (1992) and the *S. pombe* sequence was previously reported by Good et al., (1997b). The folding was performed at a temperature of 30°C and the most stable estimates (highest change in free energy) were chosen.

2.Preparation of ribosome precursor RNA

An XbaI restriction fragment of *S. pombe* rDNA containing 276 bps of the 5'ETS and 140 bps of the 18S rRNA was cloned in the PTZ19R plasmid (pSp5'ETS 270, Fig. 2.1) and a template containing this sequence, preceded with a T_7 RNA polymerase promoter was prepared by PCR amplification using primers specific for the promoter and the 5' end of the 18S rRNA sequence. The pre-rRNA was expressed using this 439 bps template and bacteriophage T_7 RNA polymerase as previously described (Melton et al., 1984; Lee and Nazar, 1997). The precursor transcript was purified on a 6% denaturing polyacrylamide gel and labelled at the 5' end using bacteriophage T_4 polynucleotide kinase and [γ-^{32}P] ATP (Chaconas and van der Sande, 1980) after dephosphorylation with calf intestinal alkaline phosphatase (Sambrook et al., 1982). The dephosphorylated RNA was purified on a 6% denaturing polyacrylamide gel before the RNA was labelled and the labelled RNA was again purified on a 6% (w/v) denaturing polyacrylamide gel.

3.Structure analysis by limited ribonuclease digestion.

RNA structure was probed by partial nuclease digestion essentially as described by

Van Ryk and Nazar, (1992). The *in vitro* transcribed and labelled 5' ETS precursor RNA was dissolved and renatured in physiological-like buffer (0.2 M KCl, 50 mM $MgCl_2$, 10 mM Tris, pH 7.5) or dissolved in denaturing buffer (7 M urea, 1 mM EDTA, 20 mM Na citrate, pH 5.0). Ten µl aliquots, containing 10 µg of carrier RNA (*Torula*) were incubated for ten minutes in the presence of different amounts of enzyme at 0°C in physiological-like buffer or 50°C in denaturing buffer. Digestion was terminated by the addition of 200 µl of 0.1% SDS, 0.3 M NaOAc, pH 5.1 followed immediately with 200 µl of phenol:chloroform (50/50:v/v). Each sample was mixed by vortex for two minutes, the phases were separated by brief centrifugation in a microfuge and the RNA in the aqueous layer was precipitated with 2.5 volumes of ethanol. The digested RNA pellets were dissolved in 10 µl of ddH_20, 10 µl of loading buffer (formamide containing 0.5% xylene cyanol and 0.05% bromphenol blue) was added; the solution was heated for three minutes at 65°C and applied to an 8% (w/v) polyacrylamide sequencing gel. After fractionation at 1200V for four-six hours, the fragments were detected by autoradiography.

C. Results

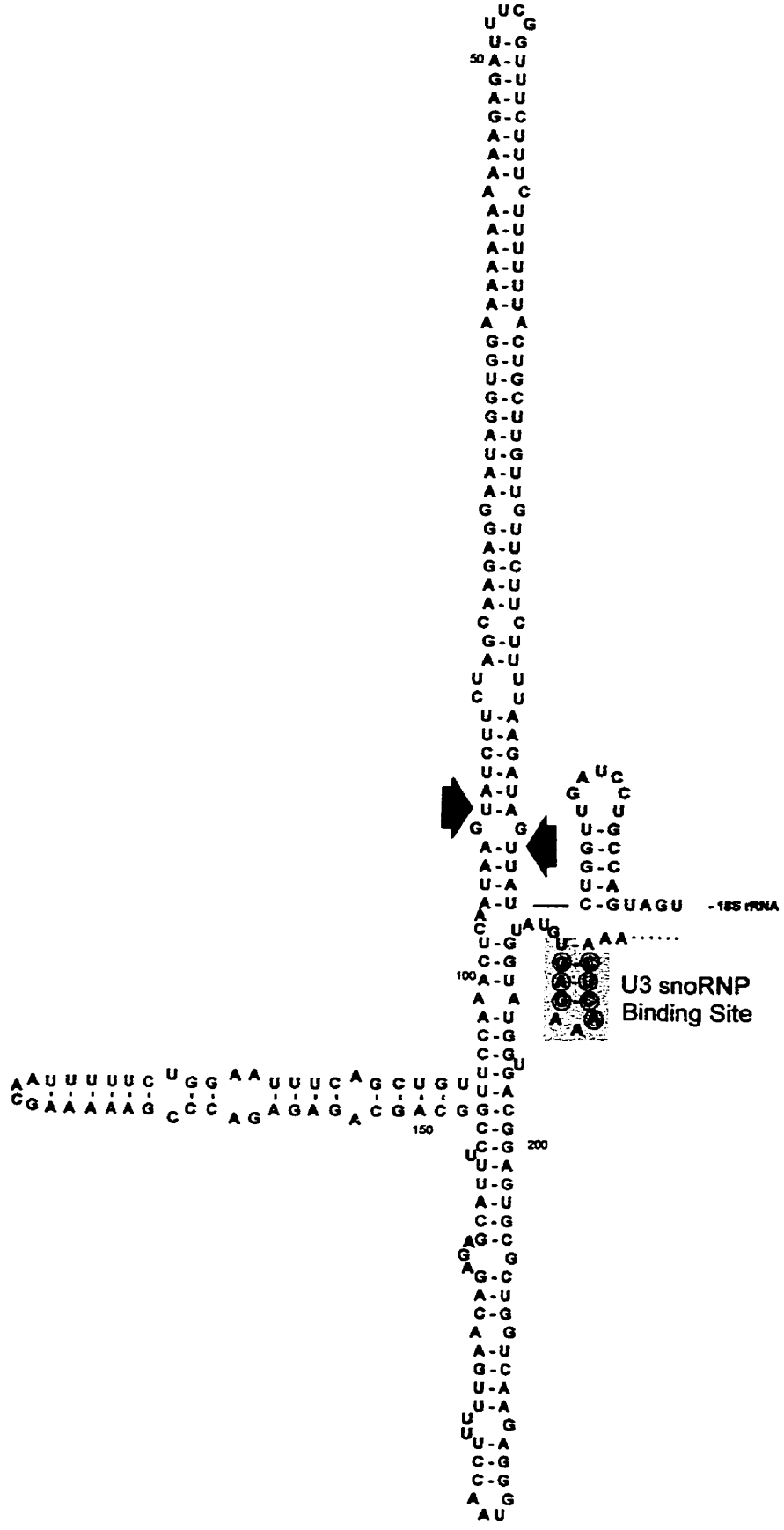
In their structural analysis on the 5'ETS in *S. cerevisiae*, Yeh and Lee, (1992) attempted to generalize their model to include more diverse yeasts such as *S. pombe*. Unfortunately, as shown in Figure 4.1, to retain the putative basic features which they described in their estimate of the structure in *S. cerevisiae*, the region immediately upstream of the mature 18S rRNA was presented as a rather open structure with a relatively high number of G-U base-pairs in critical regions. Using updated computer analyses with the mfold web server (http://www.ibc.wustl.edu/~zuker/rna/form1.gci) which is based on the algorithms of Zuker and coworkers (Walter et al., 1994), the 270 bases most proximal to the 18S rRNA sequences were re-examined in *S. pombe* with a substantially different conclusion. As shown in Figure 4.2B, the new estimate for the same region clearly is base-paired more extensively and is folded primarily into three long hairpins (I-III, respectively) forming a "crucifix"-like structure. More important, this estimate was entirely consistent with the patterns of partial nuclease digestion.

For this study, the precursor RNA was prepared which contained the 276 base-pairs of 5'ETS sequence together with the first 60 bps at the 5'-end of the mature 18S rRNA. The RNA was expressed with bacteriophage T_7 RNA polymerase and labelled at the 5'-end with T_4 polynucleotide kinase. The purified and renatured RNA precursor was then digested with a range of nuclease concentrations to clearly differentiate primary and secondary cleavage sites. As illustrated in the example analyses shown in Figure 4.3 and summarized in Figure 4.2B, when the structure was probed by limited RNase A, S_1 or T_1 nuclease digestion, the primary cleavage sites were consistent with the new

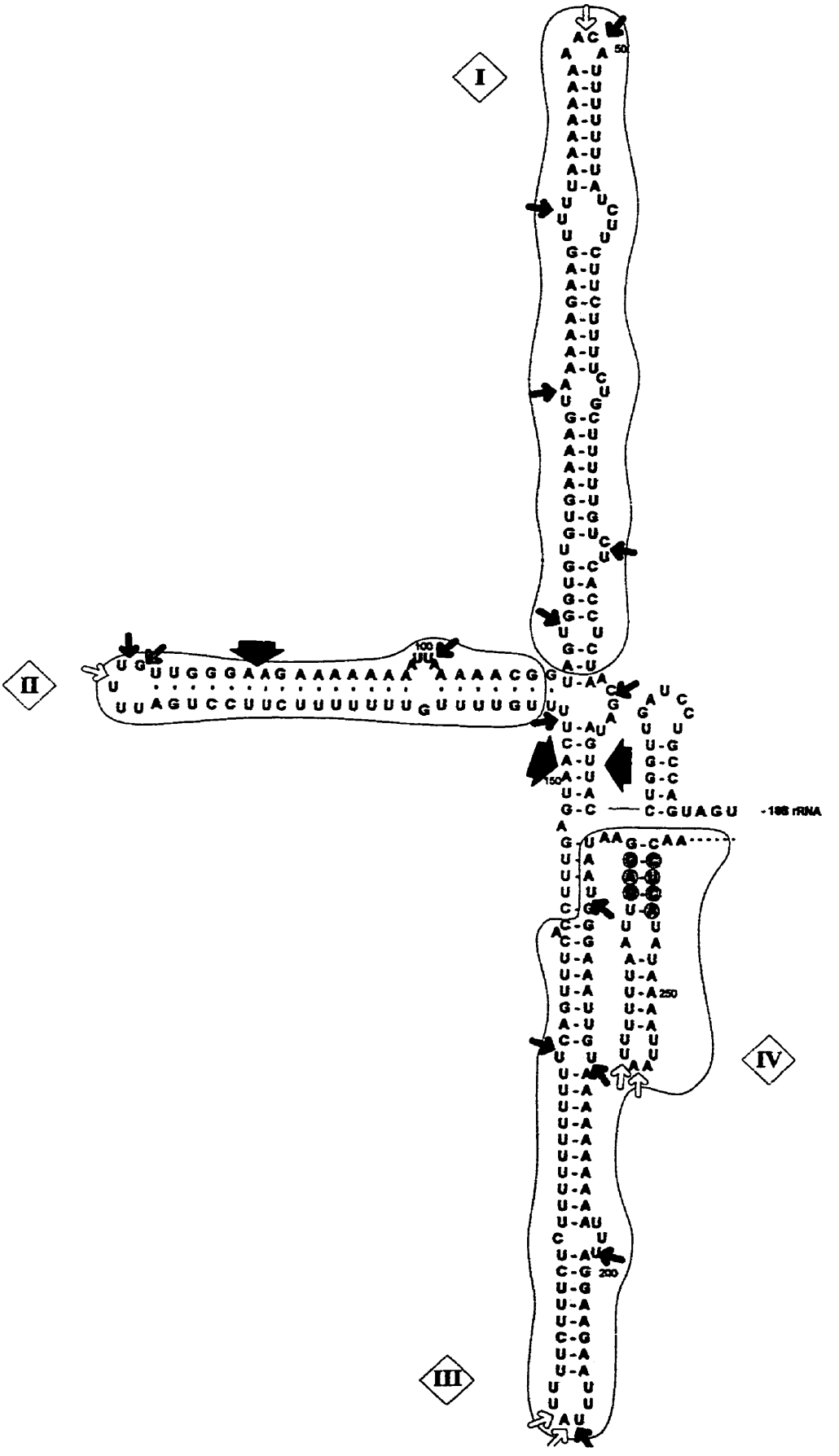
Figure 4.2. A comparison of revised estimates for the proximal region of the 5'ETS sequences in yeast 35S pre-rRNA precursors.

The estimates were based on the present study in *S. pombe* and predicted using the mfold web server (http://www.ibe.wustl.edu/~zuker/rna/forml.cgi). The sequence for *S. cerevisiae* (A) was taken from Yeh and Lee, (1992) and *S. pombe* (B) from Good et al., 1997. Lightly shaded areas represent the mature 18S rRNA. The large arrowheads represent known cleavages during RNA maturation (Hughes and Ares, 1991; Good et al., 1997b) and the shaded residues are common to both models and are part of the U3 snoRNA binding site in *S. cerevisiae* (Beltrame and Tollervey, 1995). The arrows indicate the results of limited digestion with pancreatic (black), S_1 (open) and T_1 (grey) ribonuclease as illustrated in Figure 3.3. The large shaded regions indicate the bases that were deleted as part of the analysis in chapter three: helix 1(dI), helix II (dII) and helices III & IV (dIII).

A.



B.

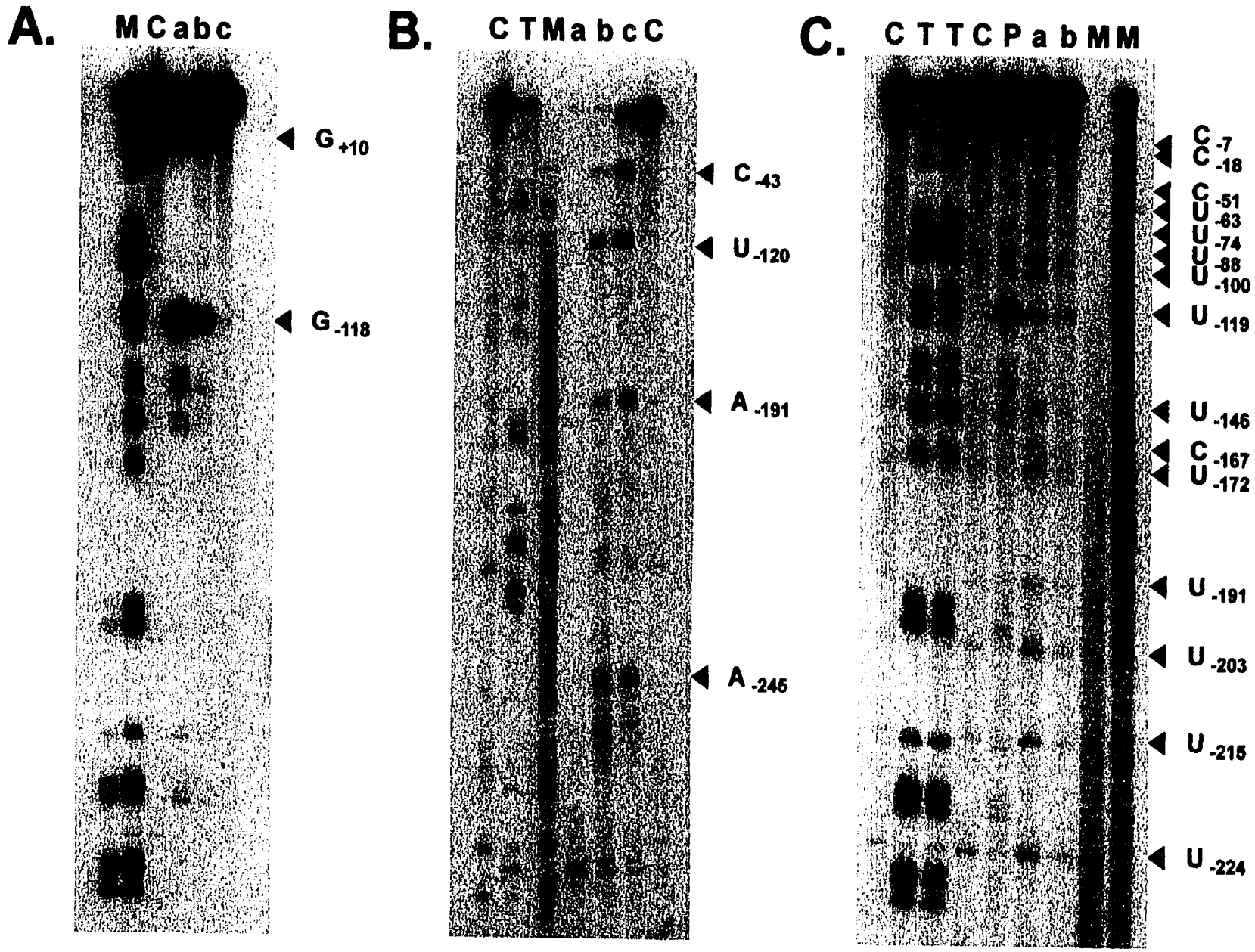


secondary structure estimate. Where S1 nuclease was used the cleavage sites (C-43, U-120, A-191 and A-245) were restricted to the ends of the hairpin structures. When RNase A was utilized, cleavages were evident at the ends of the hairpin structures as well as at some of the internal unpaired bulges (eg. U-203, U-100 and U-74). The differences between the two models were especially clear with T_1 ribonuclease digestion. As indicated by the many arrows in Figure 4.1, the earlier estimate by Yeh and Lee (1992) predicts that numerous guanylic acid residues in the *S. pombe* 5'ETS would be susceptible to this nuclease. However, much more consistent with the new estimate, only one guanylic acid residue (G_{118}), at the end of the second hairpin was readily cleaved (Fig. 4.3A) in this highly base-paired structure. This extensive base-pairing was equally evident with S_1 nuclease (Fig. 4.3B,) where the primary sites of cleavage were entirely restricted to the ends of the three extended hairpin structures and the shorter hairpin which precedes them. Also, with RNase A, the primary sites of cleavage were again restricted to either the ends of the extended hairpins or internal bases that were predicted to be unpaired (Fig. 4.3C).

In view of these contrasting estimates for the proximal 5'ETS structure in *S. pombe*, the structure in *S. cerevisiae* was further examined using the same updated software. As also shown in Figure 4.2A, while the actual sequences are substantially different, the equivalent region in each case is highly paired and folded into three extended hairpins.

Figure 4.3. Nuclease-sensitive sites in the proximal region of the 5'ETS sequence in *S. pombe* 35S pre-rRNA.

The RNA was transcribed, labelled and partially digested under physiological-like (a-c) or denatured (M and T) conditions as described in Materials and Methods. The digests were fractionated on denaturing 8% (w/v) polyacrylamide gels with the fragments detected by autoradiography. The RNA (consisting of 200,000 cpm labelled RNA and 20 ug of carrier RNA) was digested with 0.02 (a), 0.2 (b) or 1 (c) units of T_1 ribonuclease (A), 0.05(a) 0.25(b) or 1.25(c) units of S_1 nuclease (B)or 10^{-4} (a) or 10^{-5} units per mg of RNase A. Digests under denatured conditions with base (M), T_1 ribonuclease (T) or T1 ribonuclease under physiological conditions (P) were used to identify the cleavage positions; a sample incubated in the absence of enzyme is included on each gel (C).


A.
MCabc
G +10
G -118
B.
CTMabcC
C -43
U -120
A -191
A -245
C.
CTTCPabMM
C -7
C -18
C -51
U -63
U -74
U -88
U -100
U -119
U -146
C -167
U -172
U -191
U -203
U -215
U -224

D. Discussion

The secondary structure analysis presented here, raises further questions about the structure of this spacer region and its relationship to trans-acting factors such as the U3 snoRNP. As indicated in the structure comparison presented in Figure 4.2, the proximal 5'ETS regions in *S. cerevisiae* and *S. pombe* share more structural similarities than the previous models have predicted. While the *S. cerevisiae* model differs from that proposed by Yeh and Lee, (1992) (Fig. 4.1A) it is more consistent with newer estimates for this region that have been reported (Venema et al., 1995b, Abou Elela et al., 1996). In addition to the three hairpin structures and the conserved small hairpin at the 5' end of the 18S rRNA, two features seem particularly important with respect to rRNA processing. As indicated by the bold arrowheads, in both RNAs, two key cleavages occur on opposite sides of the first hairpin within a helical stem. These are consistent with RNase III-like endonuclease activities which also have been well documented for the 3'ETS region in the same two organisms (Abou Elela et al., 1996; Rotondo et al., 1997).

Perhaps most importantly, the new structures reveal an intriguing feature which may be linked to the action of the U3 snoRNA. Two studies in *S. cerevisiae* (Beltrame and Tollervey, 1992; Beltrame et al., 1994) have indicated that a UGAGAAACUC sequence (U_{+217}-C_{-226}) is critical to an interaction with the U3 snoRNA and, complementary mutations have indicated a 10 base-pair helix forms between them (Beltrame and Tollervey, 1995). Sequence comparisons in *S. pombe*, however, have not revealed an obvious equivalent sequence or even comparable sequence complementarity with the U3 snoRNA. In this study, the structure comparison in Figure 4.2 nevertheless does reveal an interesting

equivalent feature in this region. In the comparison, the critical sequence in *S. cerevisiae* is predicted to form a short stem which is extended in *S. pombe* but actually retains seven identical residues in equivalent positions (shaded circles). As a result, although there is little sequence homology in the linear sequences, seven nucleotides are conserved in the folded sequence.Whether the U3 snoRNA acts to disrupt this structure or interacts in an as yet undefined fashion, remains unclear but the similarity in residue distribution and its close proximity to the maturing termini in the higher order structure clearly is a significant question, raised by the new structural estimates.

In conclusion, the new estimate shows that the region most proximal to the 18S rRNA of the 5' ETS in *S. pombe* pre-rRNA is far more similar to the same region in *S. cerevisiae* than what was previously believed. In addition, the new estimate shows further similarities in these two organisms with respect to the position of known processing sites and to the binding site of the U3 snoRNA.

Chapter 5. Structural elements in the 5' ETS of *S. pombe* affecting pre-rRNA processing.

A. Introduction

Many studies have revealed that structural elements of both the internal and external transcribed spacers of pre-rRNA are essential for efficient processing of this transcript. However, similar to the earlier work presented in chapter three of this thesis, most studies have utilized large deletions that remove a significant number of bases and generally one or more extended helices. The secondary structural estimate for the 5'ETS of *S. pombe* presented in chapter four, revealed several interesting features in terms of the position of known processing sites within this structure as well as a potentially conserved region that may be related to the action of the U3 snoRNP (Fig. 4.2B).

In order to pinpoint essential features of the structure presented in chapter four, PCR-based mutagenesis was used to introduce base substitutions into helices II, III and IV that would disrupt the local secondary structure. An initial set of mutations revealed that the structure or sequence at the A0' site and the putative U3 binding site were essential for 18S rRNA maturation. As well, disruption of the secondary structure at the end of helix III dramatically reduced 18S rRNA maturation. A further series of mutations was then constructed in order to determine whether the primary sequence or the secondary structure were the important features in these areas. In each case the result was different. The putative U3 snoRNA binding site is dependent on sequence, the region at the end of helix III is dependent on structure and the A0' processing site may be dependent on both. The relationship of these results to what has been previously reported is discussed.

B. Materials and Methods

Most methods utilized in this chapter have been previously described. For details on PCR conditions, northern hybridization analysis, low molecular weight rRNA analysis, copy number determinations and growth characteristics of transformants generated in this study refer to chapter two, sections: 4(a), 5(b), 5(c),4(h) and 2 respectively.

1. Confirmation of transformant colonies.

The presence of mutations in transformant colonies were confirmed by sequencing. Initially, a PCR amplification was performed from a yeast colony using the 18S rRNA tag specific primer and an upstream primer as described in chapter three. The nucleotide sequence of the resultant product from this reaction was determined by cycle sequencing. An appropriate primer was 5' end labeled with T_4 polynucleotide kinase as described in chapter two, section 4(e). Approximately 25 ng of labeled primer was added to; 4.5 ul of Taq polymerase buffer, 2.5 units of Taq enzyme and ddH_20 to give a total volume of 36 ul of pre-reaction mix. 8 ul of this pre-reaction mix was added to tubes containing 2 ul of either G, A, T or C standard dideoxy termination mixes. The tubes were cycled under the following conditions: 95°C four minutes, then 25 cycles of 95°C one minute, 50°C one minute and 72°C one minute The reaction products were then separated on 6% denaturing polyacrylamide gels and exposed to X-ray film for autoradiography.

C. Results

Two of the deletions examined in chapter three dII and dIII removed loops II and III plus IV respectively. In both cases, these mutations resulted in the abolishment of mature 18S rRNA production. To further examine the importance of the structural features revealed in the analysis presented in chapter four, substitution mutants were prepared using a PCR-mediated mutagenesis strategy (Good and Nazar, 1992) in which base substitutions were introduced to disrupt the local secondary structure. Each mutant rDNA was expressed *in vivo* and, as previously described (chapter two), a neutral marker in the 18S rRNA was used to measure the amount of mature 18S rRNA which was derived from the mutant genes. As shown in Figures 5.1-5.6, and summarized in table 5.1, when a marker-specific probe was used for hybridization analyses, all normal plasmid controls (Ctl) indicated substantial amounts of mature plasmid-derived 18S rRNA and no tagged RNA was detected in extracts of untransformed cells (Wt). The amount of tagged RNA, however, was very different with the substitution mutants. In helix II, changes at the base of the stem (IId) or in the loop end (IIa) had no effect but changes at the intermediate cleavage site had dramatic effects (IIb and IIc) (Fig. 5.1). Indeed, a four base substitution immediately adjacent to the cleavage site was sufficient to fully inhibit 18S rRNA maturation.

A specific change in the end of helix III also, was observed to have dramatic effects (Fig 5.2). Most substitutions in helix III (IIIa-IIIC) had little or no effect with 88-100% normal levels of 18S rRNA being produced. However, a three base substitution near the end of the large hairpin (IIId) reduced the plasmid derived 18S rRNA level by 69%. Most important, a change in the putative U3 snoRNA (IVb) binding site also was observed to be

Figure 5.1. Expression of plasmid-derived 18S rRNA in *S. pombe* cells containing substitutions in helix II of the 5'ETS sequence.

Helix II (Fig. 4.2B) is diagrammatically represented and the bases in the ellipses were substituted with the bases in the shaded ellipses that are connected by the dotted line. Whole cell RNA was prepared from *S. pombe* cells transformed with mutant rRNA genes and fractionated on agarose/formaldehyde gels (Good et al., 1994; 1997a). RNA from untransformed *S. pombe* cells (Wt) and cells transformed with normal but "tagged" (Ctl) *S. pombe* rDNA (Good et al., 1997a) was included on each gel. Gels were blotted on nylon and hybridized (Rose et al., 1990) with a ^{32}P-labelled oligomer, specific for the tagged 18S rRNA transcribed from the plasmid-associated rDNA (Good et al., 1997a). The results with three individual transformants I, II, III are presented. Position of the mature 18S rRNA is indicated on the right and the percentage of 18S rRNA in transformant colonies is given below each result. For experimental errors related to each of these results refer to table 5.1.

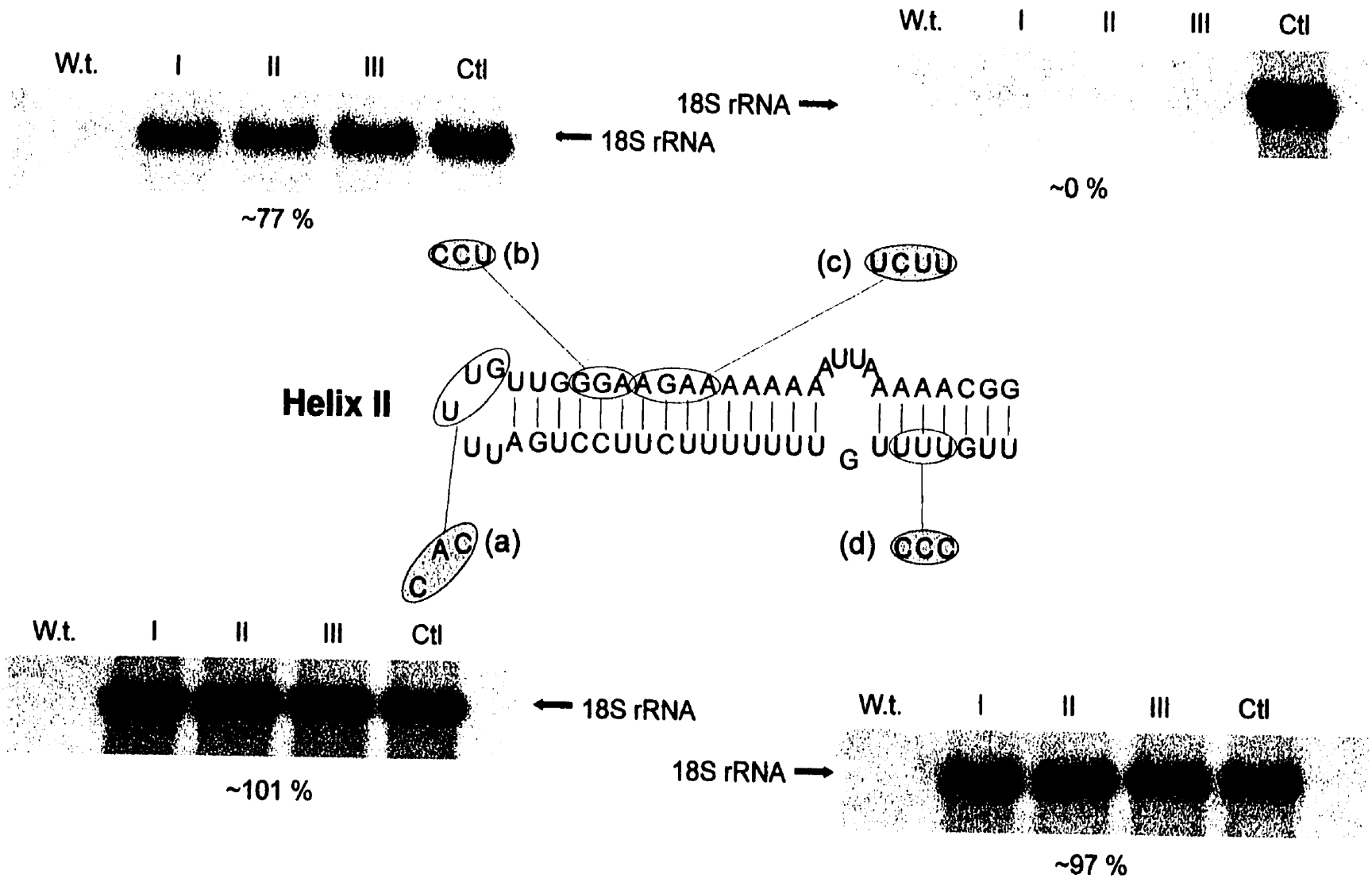
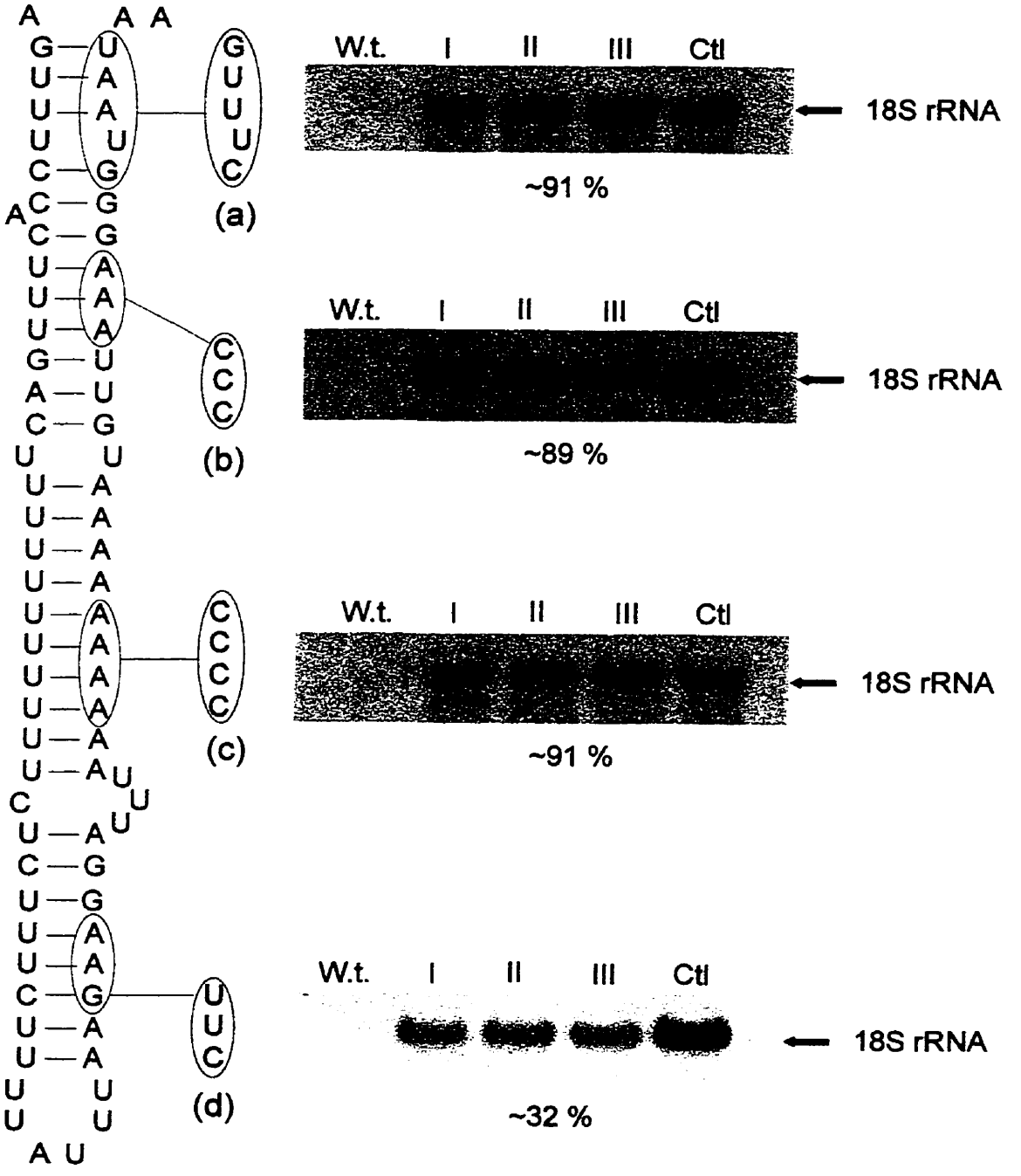

W.t.
I
II
III
Ctl
18S rRNA
~77 %
W.t.
I
II
III
Ctl
18S rRNA
~0 %
CCU (b)
(c) UCUU
Helix II
UG
UUGGGAAGAAAAAAA
AUUA
AAAACGG
U
UUAGUCCUUCUUUUUUU
G
UUUUGUU
AC
C
(a)
(d) CCC
W.t.
I
II
III
Ctl
18S rRNA
~101 %
W.t.
I
II
III
Ctl
18S rRNA
~97 %

Figure 5.2. Expression of plasmid-derived 18S rRNA in *S. pombe* cells containing substitutions in helix III of the 5'ETS sequence.

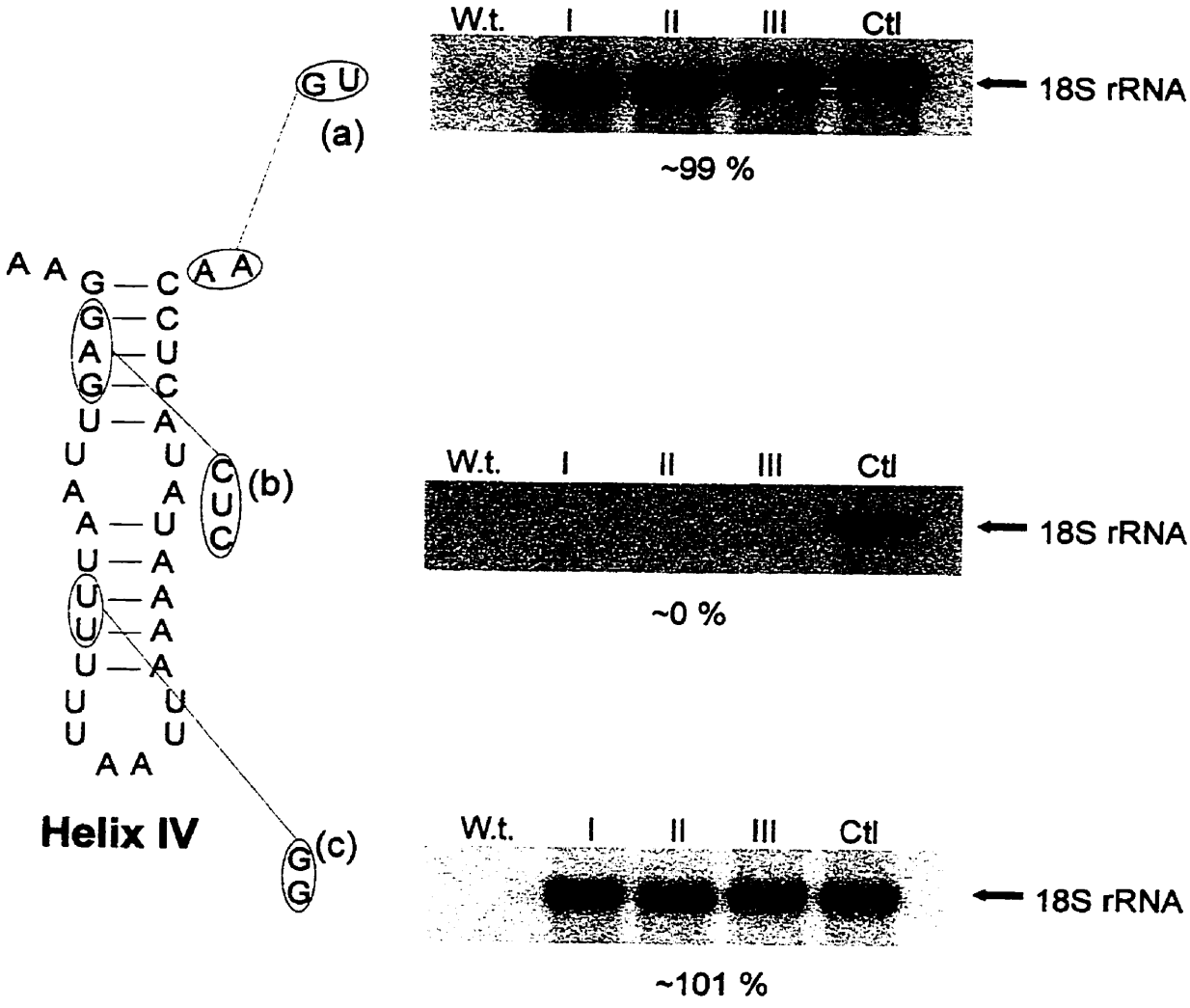
Helix III (Fig 4.2B)is diagrammatically represented and the bases in the ellipses were substituted with the bases in the shaded ellipses that are connected by the dotted line. Whole cell RNA was prepared from *S. pombe* cells transformed with mutant rRNA genes and fractionated on agarose/formaldehyde gels (Good et al., 1994; 1997a). RNA from untransformed *S. pombe* cells (Wt) and cells transformed with normal but "tagged" (Ctl) *S. pombe* rDNA (Good et al., 1997a) was included on each gel. Gels were blotted on nylon and hybridized (Rose et al., 1990) with a ^{32}P-labelled oligomer, specific for the tagged 18S rRNA transcribed from the plasmid-associated rDNA (Good et al., 1997a). The results with three individual transformants I, II, III are presented. Position of the mature 18S rRNA is indicated on the right and the percentage of 18S rRNA in transformant colonies is given below each result. For experimental errors related to each of these results refer to table 5.1.



Helix III

Figure 5.3. Expression of plasmid-derived 18S rRNA in *S. pombe* cells containing substitutions in helix IV of the 5'ETS sequence.

Helix IV (Fig 4.2B) is diagrammatically represented and the bases in the ellipses were substituted with the bases in the shaded ellipses that are connected by the dotted line. Whole cell RNA was prepared from *S. pombe* cells transformed with mutant rRNA genes and fractionated on agarose/formaldehyde gels (Good et al., 1994; 1997a). RNA from untransformed *S. pombe* cells (Wt) and cells transformed with normal but "tagged" (Ctl) *S. pombe* rDNA (Good et al., 1997a) was included on each gel. Gels were blotted on nylon and hybridized (Rose et al., 1990) with a ^{32}P-labelled oligomer, specific for the tagged 18S rRNA transcribed from the plasmid-associated rDNA (Good et al., 1997a). The results with three individual transformants I, II, III are presented. Position of the mature 18S rRNA is indicated on the right and the percentage of 18S rRNA in transformant colonies is given below each result. For experimental errors related to each of these results refer to table 5.1.


W.t.
I
II
III
Ctl
18S rRNA
~99 %
(a)
(b)
(c)
~0 %
~101 %
Helix IV

critical, with no plasmid derived 18S rRNA being observed while other mutations in this helix had no effects on maturation (IVa and IVc) (Fig. 5.3).

In order to determine whether the primary or secondary structures were the essential features in the areas that showed dramatic effects on 18S rRNA maturation described above further substitution mutations were generated. A complementary mutation to IIc (IIe) and a mutation which disrupted the structure of the helix on the opposite side (IIf) gave identical results showing that no 18S rRNA was produced (Fig. 5.4). These results indicate that the sequences at the A0' site are essential for 18S rRNA maturation and they suggest that these bases may have to be in a double strand context. In contrast, the region at the end of helix III is dependent only on the secondary structure. A complementary mutation which restored the base pairing in this helix (IIIf) also fully restored the production of mature 18S rRNA. Whereas, when a mutation that disrupted the other side of the helix (IIIe) was introduced, the levels of 18S rRNA production were decreased again to approximately 35% of the control (Fig. 5.5).The results of the putative U3 snoRNA binding site gave different results again. Restoration of base pairing by a complementary mutation (IVe), did not restore 18S rRNA production which suggests, that the GAG sequence substituted in mutation IVb are essential. In contrast, the substitution of the conserved bases on the other side of the helix (IVd) did not effect the levels of 18S rRNA production (Fig. 5.6).

As previously shown in chapter three, there is a strong interdependence in the production of the SSU and LSU rRNAs when large deletion mutations were constructed in the 5' ETS of *S. pombe* pre-rRNA. Therefore, maturation of the LSU rRNAs were also examined here. As previously described (Abou Elela et al., 1995), the maturation of

Figure 5.4. Expression of plasmid-derived 18S rRNA in *S. pombe* cells containing substitutions in helix II of the 5'ETS sequence to determine if the primary or secondary structure is the critical element.

Helix II (Fig 4.2B) is diagrammatically represented and the bases in the ellipses were substituted with the bases in the shaded ellipses that are connected by the dotted line. Whole cell RNA was prepared from *S. pombe* cells transformed with mutant rRNA genes and fractionated on agarose/formaldehyde gels (Good et al., 1994; 1997a). RNA from untransformed *S. pombe* cells (Wt) and cells transformed with normal but "tagged" (Ctl) *S. pombe* rDNA (Good et al., 1997a) was included on each gel. Gels were blotted on nylon and hybridized (Rose et al., 1990) with a ^{32}P-labelled oligomer, specific for the tagged 18S rRNA transcribed from the plasmid-associated rDNA (Good et al., 1997a). The results with three individual transformants I, II, III are presented. Position of the mature 18S rRNA is indicated on the right and the percentage of 18S rRNA in transformant colonies is given below each result. For experimental errors related to each of these results refer to table 5.1.

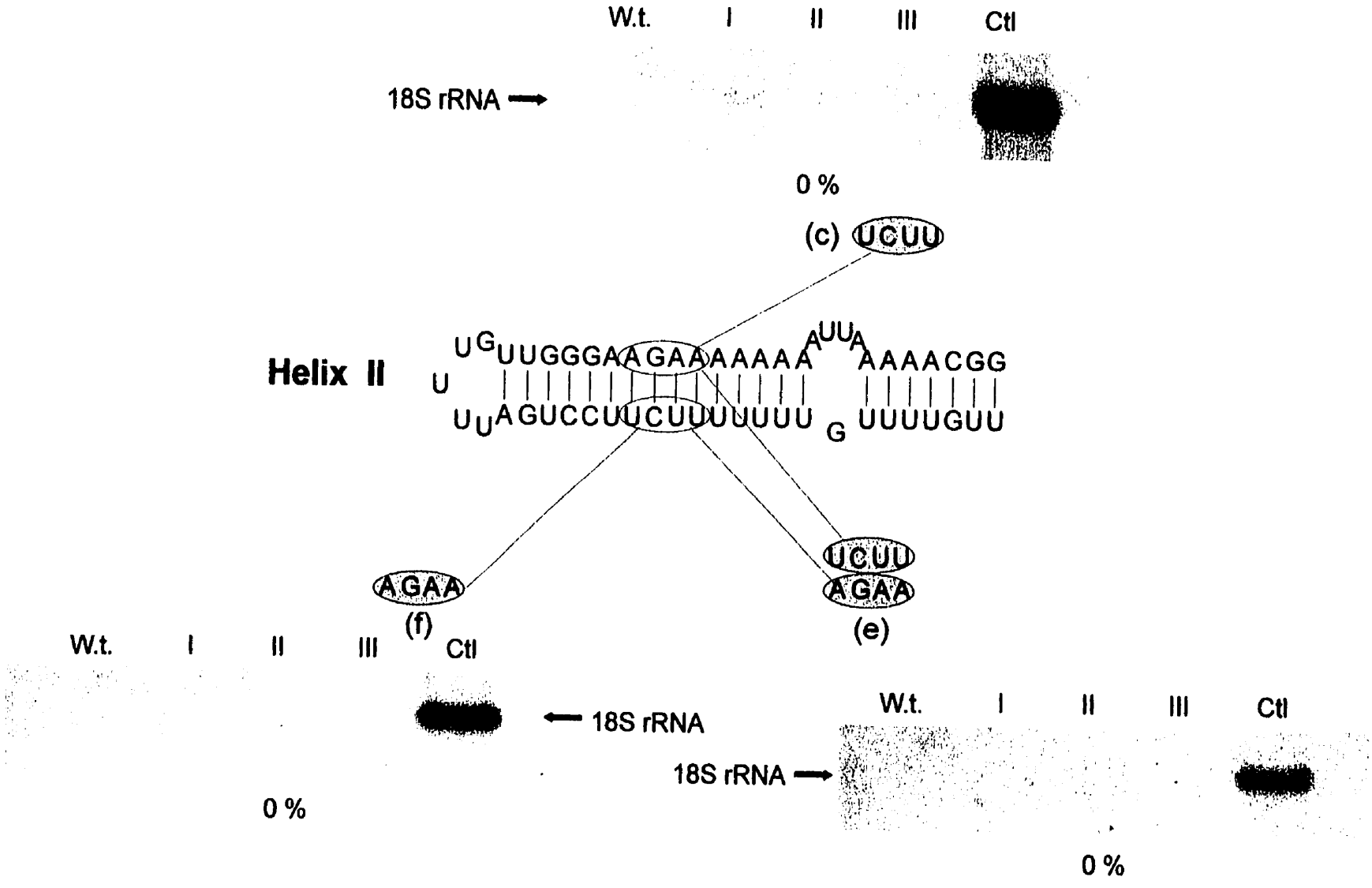

W.t.
I
II
III
Ctl
18S rRNA
0 %
(c) UCUU
Helix II
UGUUGGGAAGAAAAAAAUUAAAAACGG
UUAGUCCUUCUUUUUUUGUUUUGUU
AGAA
(f)
UCUU
AGAA
(e)
W.t.
I
II
III
Ctl
18S rRNA
0 %
W.t.
I
II
III
Ctl
18S rRNA
0 %

Figure 5.5. Expression of plasmid-derived 18S rRNA in *S. pombe* cells containing substitutions in helix III of the 5'ETS sequence to determine if the primary or secondary structure is the critical element.

Helix III (Fig 4.2B) is diagrammatically represented and the bases in the ellipses were substituted with the bases in the shaded ellipses that are connected by the dotted line. Whole cell RNA was prepared from *S. pombe* cells transformed with mutant rRNA genes and fractionated on agarose/formaldehyde gels (Good et al., 1994; 1997a). RNA from untransformed *S. pombe* cells (Wt) and cells transformed with normal but "tagged" (Ctl) *S. pombe* rDNA (Good et al., 1997a) was included on each gel. Gels were blotted on nylon and hybridized (Rose et al., 1990) with a ^{32}P-labelled oligomer, specific for the tagged 18S rRNA transcribed from the plasmid-associated rDNA (Good et al., 1997a). The results with three individual transformants I, II, III are presented. Position of the mature 18S rRNA is indicated on the right and the percentage of 18S rRNA in transformant colonies is given below each result. For experimental errors related to each of these results refer to table 5.1.

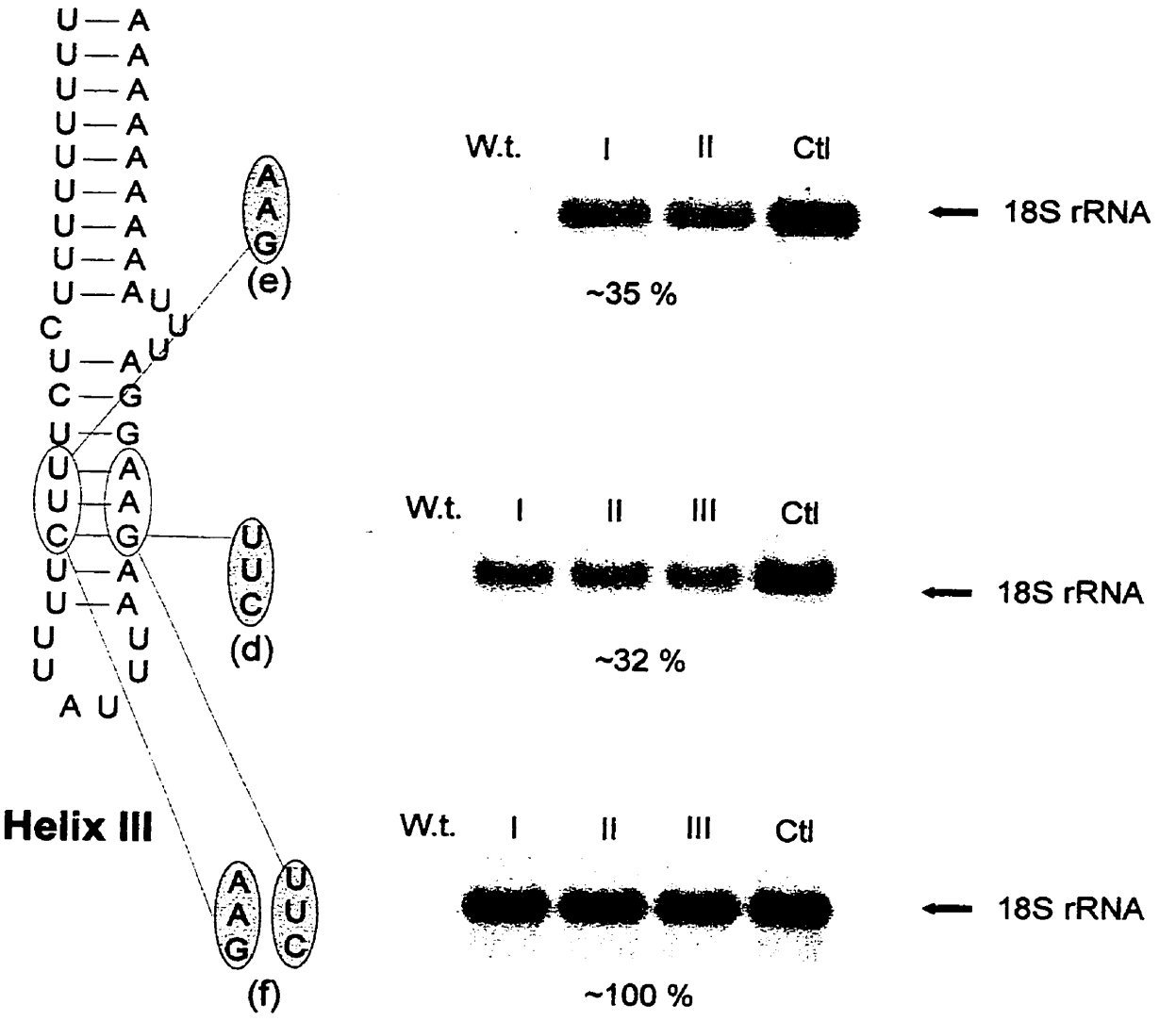
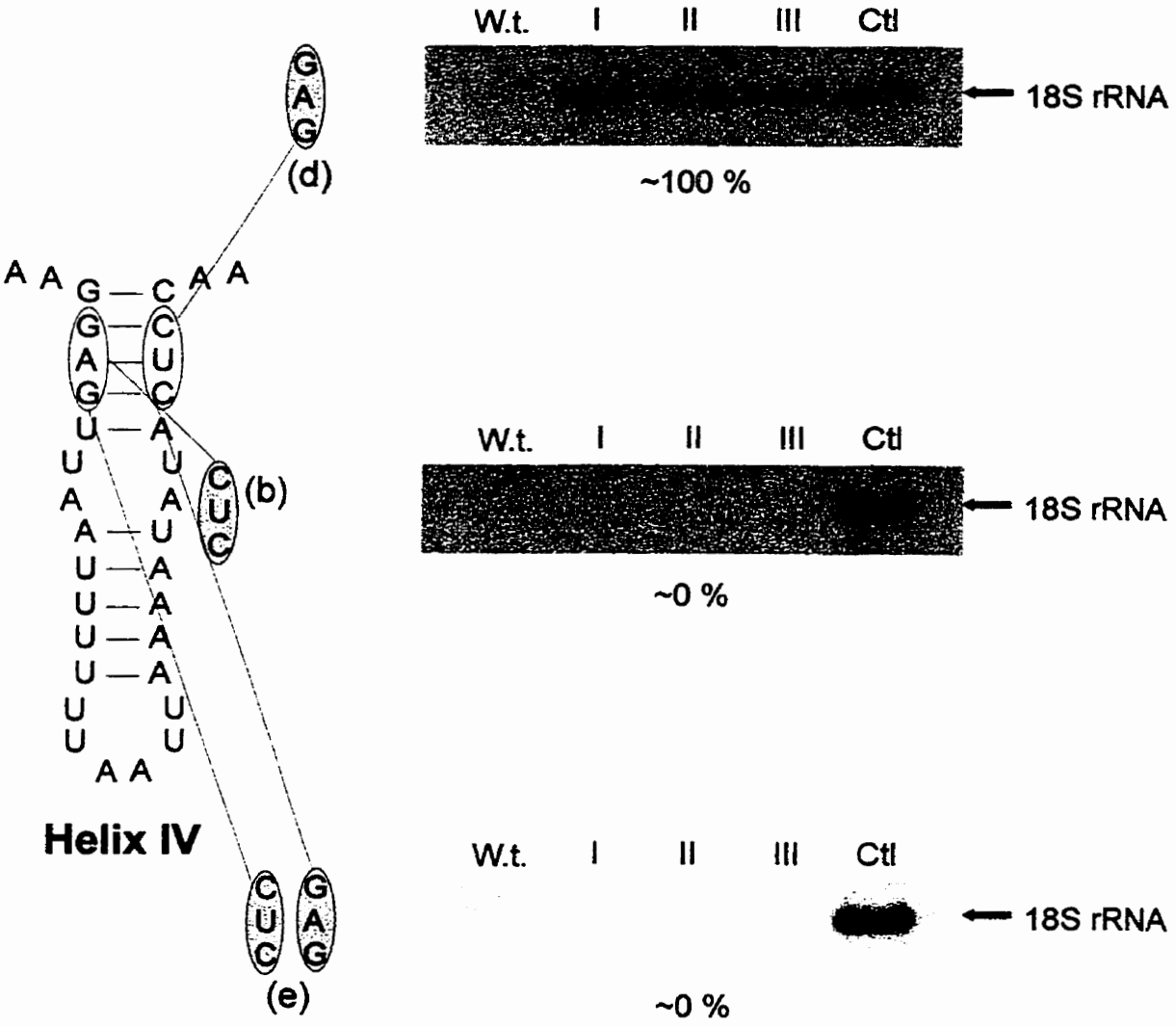

Helix III
(e)
(d)
(f)
W.t.
I
II
III
Ctl
18S rRNA
~35 %
~32 %
~100 %

Figure 5.6. Expression of plasmid-derived 18S rRNA in *S. pombe* cells containing substitutions in helix IV of the 5'ETS sequence to determine if the primary or secondary structure is the critical element.

Helix IV (Fig 4.2B) is diagrammatically represented and the bases in the ellipses were substituted with the bases in the shaded ellipses that are connected by the dotted line. Whole cell RNA was prepared from *S. pombe* cells transformed with mutant rRNA genes and fractionated on agarose/formaldehyde gels (Good et al., 1994; 1997a). RNA from untransformed *S. pombe* cells (Wt) and cells transformed with normal but "tagged" (Ctl) *S. pombe* rDNA (Good et al., 1997a) was included on each gel. Gels were blotted on nylon and hybridized (Rose et al., 1990) with a ^{32}P-labelled oligomer, specific for the tagged 18S rRNA transcribed from the plasmid-associated rDNA (Good et al., 1997a). The results with three individual transformants I, II, III are presented. Position of the mature 18S rRNA is indicated on the right and the percentage of 18S rRNA in transformant colonies is given below each result. For experimental errors related to each of these results refer to table 5.1.


W.t.
I
II
III
Ctl
18S rRNA
~100 %
(d)
(b)
(e)
Helix IV
~0 %
~0 %

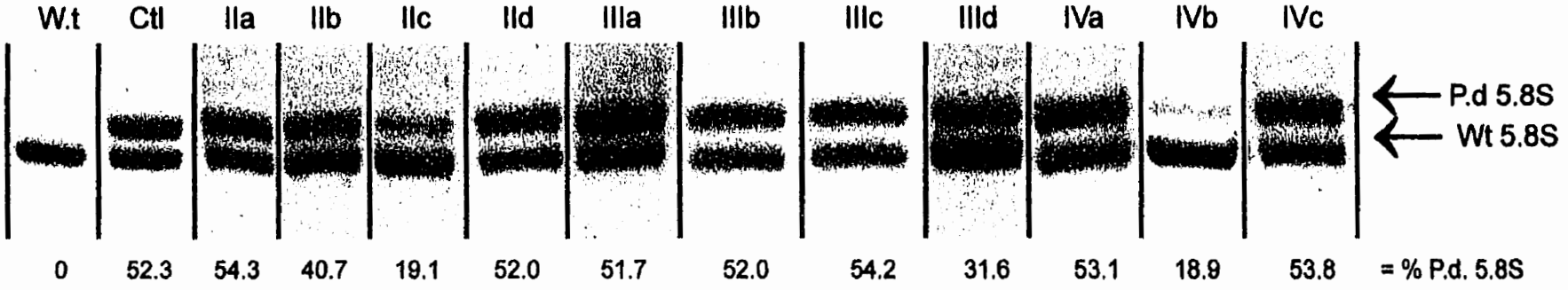
plasmid-derived 5.8S rRNA (labelled plasmid-derived in Figs. 5.7) could be quantified accurately through the use of a neutral four base insertion which results in a distinct electrophoretic mobility. Under the growth conditions which were used in this study, approximately 52% of the mature 5.8S rRNA is plasmid-derived. As can be seen in Fig. 5.4 and summarized in table 5.1, mutations which did not have an effect on 18S rRNA production (IIa, IId, IVa and IId), also did not effect the maturation of the 5.8S rRNA and in all cases control amounts were observed. Where mutations had a 10% or less effect on 18S rRNA production (IIIa, IIIa and IIIC), no effect on the production of 5.8S rRNA was again observed. However, effects on the maturation of the 5.8S rRNA were seen in the cases where introduction of a mutation resulted in at least a 20% decrease in 18S rRNA production. In mutant IIb a decrease in 18S rRNA by approximately 25% was coupled with a 20% reduction in 5.8S rRNA. Also, mutants IIId and IIIa were shown to have 5.8S rRNA levels reduced to almost 60% of control levels. Consistent with what was reported in chapter three, when a mutation reduced the levels of 18S rRNA to below detectable levels (IIc, IIa, IIa, IVb and IID) the amount of 5.8S rRNA was reduced to approximately 40% of the control level. Therefore, as seen in the earlier studies on the ITS regions (Good et al., 1997), and in chapter three of this thesis, a significant interdependence was observed between 18S rRNA maturation and the processing of the large subunit RNAs.

Finally, in order to ensure that the differences in 18S and 5.8S rRNA yields were not the result of effects on plasmid replication, the relative plasmid copy number and growth rates were examined in each transformant as described in chapter two. As shown in table 5.1, all of the values obtained were within experimental error of the control and the results

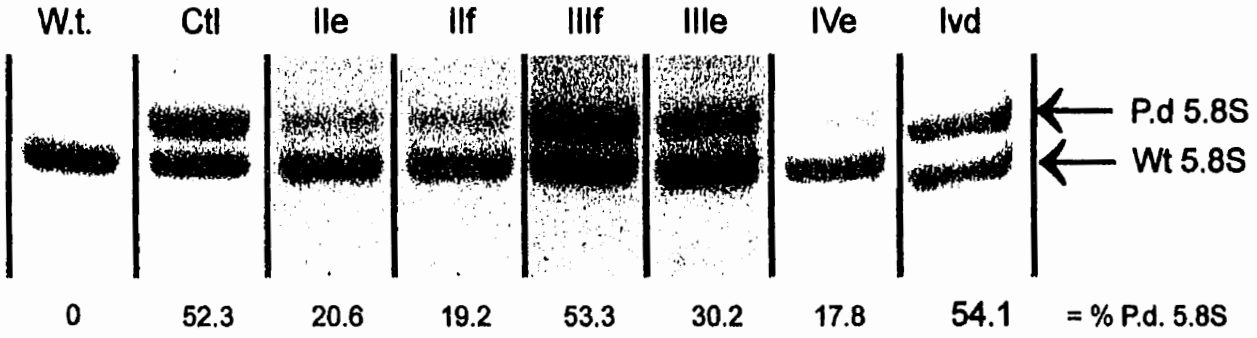
Figure 5.7. Expression of plasmid-derived 5.8S rRNA in *S. pombe* cells containing base substitutions in helices II, III and IV.

Low molecular weight RNA was prepared from *S. pombe* cells, transformed with mutant rRNA genes containing base substitutions in helices II III or IV, and fractionated on a polyacrylamide gel (Abou Elela et al., 1995) then stained with methylene blue. The specific mutations are diagrammatically represented in Figures (5.1-5.3 and 5.5-5.7) A representative experimental result has been presented. RNA from untransformed *S. pombe* cells (Wt) and cells transformed with normal but "tagged" (Ctl) *S. pombe* rDNA (Good et al., 1997a) are also included as controls. The positions of the normal (W.t.) and tagged (P.d. RNA) 5.8S rRNAs are indicated on the right. The percentage of plasmid-derived 5.8S rRNA is given below each lane, for experimental errors refer to table 5.1 A. Representative experimental results from an initial series of mutations (refer to Figs. 5.1-5.3 for mutation details). B. Representative experimental results from a second series of mutations designed to determine whether the primary or secondary structure are the essential features required for 18S rRNA maturation (for mutation details refer to Figs. 5.5-5.7).

A.


W.t
Ctl
IIa
IIb
IIc
IId
IIIa
IIIb
IIIc
IIId
IVa
IVb
IVc
P.d 5.8S
Wt 5.8S
0
52.3
54.3
40.7
19.1
52.0
51.7
52.0
54.2
31.6
53.1
18.9
53.8
= % P.d. 5.8S

B.


W.t.
Ctl
IIe
IIf
IIIf
IIIe
IVe
Ivd
P.d 5.8S
Wt 5.8S
0
52.3
20.6
19.2
53.3
30.2
17.8
54.1
= % P.d. 5.8S

which clearly demonstrates that the differences observed are not due to altered gene copy numbers.

Table 5.1 Growth characteristics of *S.pombe* cells transformed with plasmids expressing mutations in the 5' ETS of the rRNA.

Source of RNA	Plasmid derived 18S rRNA[a]	Plasmid derived 5.8S rRNA[b]	Relative plasmid copy number[c]	Transformant doubling time[d]
Control	100	52.3 +/- 1.1	74 +/- 1.2	4.3 +/- 0.1
IIa	101.2 +/- 3.2	54.3 +/- 2.3	76 +/- 2.2	4.3 +/- 0.1
IIb	77.4 +/- 3.3	40.7 +/- 3.6	72 +/- 2.3	4.3 +/- 0.1
IIc	0	19.1 +/- 3.1	74 +/- 1.2	4.3 +/- 0.2
IId	97.2 +/- 2.8	52.0 +/- 2.2	75 +/- 2.6	4.2 +/- 0.1
IIe	0	20.6 +/- 3.1	71 +/- 2.4	4.3 +/- 0.1
IIf	0	19.2 +/- 2.4	73 +/- 1.8	4.3 +/- 0.1
IIIa	91.3 +/- 2.3	51.7 +/- 1.9	70 +/- 2.7	4.4 +/- 0.2
IIIb	89.4 +/- 4.4	52.0 +/- 1.1	74 +/- 1.1	4.2 +/- 0.1
IIIc	92.4 +/- 4.3	54.2 +/- 2.3	74 +/- 1.2	4.3 +/- 0.1
IIId	32.3 +/- 3.2	31.6 +/- 3.3	76 +/- 2.5	4.3 +/- 0.2
IIIe	35.1 +/- 4.4	30.2 +/- 1.1	74 +/- 1.1	4.3 +/- 0.1
IIIf	100 +/- 2.2	53.3 +/- 3.4	73 +/- 2.0	4.3 +/- 0.1
IVa	99 +/- 2.1	53.1 +/- 0.9	72 +/- 0.9	4.3 +/- 0.1
IVb	0	18.9 +/- 3.7	73 +/- 1.7	4.3 +/- 0.1
IVc	101 +/- 2.6	51.4 +/- 2.2	74 +/- 1.2	4.3 +/- 0.1
IVd	100 +/- 3.1	52.3 +/- 2.1	72 +/- 1.1	4.4 +/- 0.1
IVe	0	17.8 +/- 3.6	73 +/- 3.1	4.3 +/- 0.1

a,b,c,d: refer to table 2.1 for notes on these.

D: Discussion

To date, most analyses of essential structural features of the transcribed spacers in the pre-rRNA molecule have involved rather large deletions removing whole structural elements. While these reports have furthered the understanding of rRNA processing it remains unclear, for the most part, which part of a particular element is essential for efficient processing. In this study, small (two-five base) nucleotide substitutions were used to assess which part of the structural features that were reported in chapter four are most critical for 18S rRNA maturation. An initial round of mutations revealed one element in each of helices II, III and IV that was critical for 18S rRNA maturation. A second round of mutations was then constructed to pinpoint whether the primary or secondary structure was the critical element in these cases.

Five processing sites have been identified previously in the 5' ETS of *S. pombe* (Good et al., 1997b) and in chapter four, (Fig. 4.2B) one of these sites, A0', was located within helix number two. Base substitutions which disrupt the secondary structure at this site, or substitutions which restore the structure but with an altered sequence, completely inhibit 18S rRNA maturation. This shows that the sequence integrity of this processing site is critical. Since, the mutations which alter the sequence also alter the structure, it is difficult to ascertain whether the maintenance of the structure at the A0' site is also important. However, disruption of the structure immediately adjacent to the site also reduced the levels of 18S rRNA which gives an indiction that the structure at the A0' may be important as well. These findings are in contrast to what has been reported previously for the A0 site in *S. cerevisiae.* Venema et al.(1995a) reported that alteration of the sequence and structure at the A0 site had

no effect on either cleavage at A0 or on the production of 18S rRNA. The reason for these differences remain unclear, however, it is possible that the machinery that recognizes the A0 and A0' sites are different and thereby have different structural requirements for their action. The end of helix III also was shown to affect maturation of the 18S rRNA. In this case it would appear that the structural integrity of this region is of critical importance and the constitution of the primary sequence is not, so long as a similar structure is maintained. There have been several other reports which show that the maintenance of the structure near a loop end is essential for processing. Using an *in vivo* mutational analysis approach van der Sande et al. (1992) examined a partially conserved extended hairpin structure in the ITS2 region of several closely related yeast species. They reported that structural alterations by the introduction of point mutations near the loop end of the helix severely reduced processing of the 25S rRNA. More recently, it was reported that changes that disrupted the secondary structure near the loop end of the conserved extended hairpin structure in the 3' ETS of *S. pombe* had severely detrimental effects on the production of both 5.8S and 25S rRNA production (Hitchen et al., 1997). It also was shown that processing in this area may be mediated by one or more protein factors. It is possible that this region of helix III may act as a signal to recruit some as yet unidentified trans-acting factors which are essential for processing.

As previously stated, the best characterized element in the 5' ETS of any organism is the U3 snoRNP binding site in *S. cerevisiae*. Tollervey and co-workers reported that the *S. cerevisiae* U3 snoRNA binds to the pre-rRNA molecule in the 5' ETS through the formation of ten perfect base pairs upstream of the A0 site (Fig 4.2A) (Beltrame and

Tollervey, 1995). Extensive sequence homology searches have not revealed an equivalent match for the *S. pombe* U3 snoRNA and the 5' ETS, however, seven of the ten nucleotides that are present in the *S. cerevisiae* 5' ETS appear to be conserved in terms of sequence, structure and relative position in *S. pombe*. Substitution of the three bases, GAG, resulted in complete inhibition of 18S rRNA production, however, substitution of the 5' four bases, did not inhibit 18S rRNA maturation at all. Also, although the structural analysis revealed these bases to be paired, this conformation is not necessary. There are a couple of possibilities that can be envisioned to explain these results. First, there may be several contact points and together with these other contact points the GAG sequence may be necessary and sufficient for binding of the U3 snoRNA. This is supported by studies in *S. cerevisiae* where it was shown that pairing of all ten bases was not essential and furthermore, all constructs which lacked pairing in the 3' terminal GAG sequence did not produce any mature 18S rRNA (Beltrame and Tollervey, 1995). A second possibility is that although these sequences are conserved in these two organisms the sequences in *S. pombe* may be unrelated to the binding and action of the U3 snoRNP. Further studies in both organisms are necessary to determine which of these possibilities are correct.

Based on many observations of an interdependence of processing between the SSU and LSU rRNAS our lab has proposed that rRNA processing serves as a quality control mechanism which helps to ensure that ribosomes are formed correctly. In the current chapter, when mutation in the 5' ETS decreased the levels of 18S rRNA, there was an accompanying decrease in the levels of 5.8S rRNA observed. Therefore, as seen in chapter three, deletions in the 5' ETS can have negative effects on the production of the LSU rRNAs

which again supports the model that processing may have evolved as a quality control mechanism.

An alternative hypothesis that explains these observations was originally proposed by this lab as well (Nazar et al., 1987). It was theorized that the spacer sequences may act as 'biological springs' which bring the processing sites in close proximity to one another possibly to allow for efficient processing. Collectively, the observations indicate that when the integrity of the spacers are disrupted, processing can be disrupted in its entirety or at least proceeds less efficiently, thus supporting this hypothesis. The two theories are not mutually exclusive and the necessity to bring the processing sites in close proximity may be a part of the quality control mechanism.

In summary, there is at least one essential element which is necessary for efficient processing of the primary transcript in each of helices II, III and IV of the 5' ETS of *S. pombe*. In each case, it would appear that the element may be different, that is, the essential feature can be sequence dependent, structure dependent, or possibly both. Therefore it would appear that the cell has evolved several different types of safeguards which help to ensure that only correctly formed ribosomes are produced.

Chapter 6 Summary and conclusions

This thesis provides new information regarding the removal of the 5' ETS spacer from the pre-rRNA transcript in the fission yeast, *Schizosaccharomyces pombe*. During the course of this work an *in vivo* mutational analysis system (Abou Elela et al. 1995) was adapted and used extensively to evaluate the structure/function relationship of this spacer. This system is based on a cloned rDNA repeat that was "tagged" in the 5.8S rRNA gene and can be manipulated *in vitro* before being expressed efficiently in *S. pombe*. The levels of rRNA that are achieved are equal to or slightly above the endogenous rRNA that is chromosomally derived. This level of expression is advantageous for studies in rRNA function and production because the plasmid derived transcripts are detected easily, must compete directly with the normal cellular rRNAs and are not essential for cell growth. This is especially important when studying mutations that would kill the cell if it had to rely on only the plasmid derived rRNA.

When this work was initiated, all analyses suggested that defects in the processing of the 5' ETS affected only the maturation of the 18S rRNA. Therefore, in order to study processing of this spacer utilizing the *S. pombe* expression system, it was necessary to introduce a new "tag" in the 18S rRNA gene that could be detected easily. The first variable region was chosen as the site for this "tag" and as anticipated expression studies showed that the newly tagged construct was neutral with respect to rRNA processing and cellular growth. This new "tag" not only has allowed these studies to be conducted, but also, during the course of this work has proven to be useful for others where it was shown that deletions in ITS1, ITS2 (Good et al., 1997a) and the 3' ETS can have significant effects on the

maturation of the 18S rRNA.

The new construct was utilized to perform the first mutational survey of the 5' ETS sequences from any organism. Previous studies from a variety of organisms have shown that specific elements within this spacer are essential for 18S rRNA production. However, there have been no reports of a sequential analysis to delineate which structural domains are essential features. The results of this work are presented in chapter three. In order to ensure that the mutations removed specific structural domains and did not overlap, the sequences were submitted for structural modeling. Based on this model, a number of attempts were made to create these mutations by previously reported PCR-based mutagenesis strategies, but due to the highly repetitive nature of the sequences, they all resulted in heterogeneous amplifications. This necessitated a need to develop the SSOE (Intine and Nazar 1998) PCR-based mutagenesis strategy. The results of the mutational survey show that, there appear to be many structural features within the 5' ETS in *S. pombe* rRNA that are essential for processing of the primary transcript as a whole. More specifically the first and last helical domains of the spacer of *S. pombe* are not essential but the rest of the transcript contains sequences which are necessary for 18S rRNA maturation. Interestingly, all the mutations which had an effect contain at least one of the intermediate processing sites which have been previously defined (Good et al., 1997a). Further mutational analysis will be required to determine if all of these processing sites are the key features within these structural domains. The results also raise the possibility that the more distal portions of the 5' ETS may be essential for processome formation and that inhibition of this complex formation can stop or substantially inhibit processing of the pre-rRNA molecule as a whole. Studies are

currently being conducted to evaluate this hypothesis.

In an attempt to maintain key structural features of the 5' ETS modeled for *S. cerevisiae,* Yeh and Lee (1992), proposed a similar structure for *S. pombe*. However, when this was compared to the structural prediction of the whole 5' ETS included in chapter three it was observed that the previously predicted structure was far more open than suggested by the new estimate. Therefore, this area was enzymatically probed and the results are presented in chapter four. Initially, both the *S. cerevisiae* and *S. pombe* sequences were submitted for computer- based modeling predictions and while the sequences in these two organisms are very different they fold into similar structures. More importantly, the enzymatic probing studies of the *S .pombe* sequences were entirely consistent with the new structural prediction. In addition to the three hairpin structures and the conserved small hairpin at the 5' end of the 18S rRNA, two features seem particularly important with respect to rRNA processing. The A0 and A1 cleavage events occur on opposite sides of the first hairpin, which is consistent with the previously documented RNase III like endonuclease activities (Abou Elela et al., 1996, Rotondo et al., 1997). Also, when extensive sequence comparisons were performed in order to determine a U3 snoRNA binding site within the 5' ETS, no such sequence was found. However, in the folded structure seven out of ten bases of the U3 snoRNA binding site in *S. cerevisiae* was found to be conserved in *S. pombe*. Therefore, the new estimate shows that the region most proximal to the 18S rRNA of the 5' ETS in *S. pombe* pre-rRNA is far more similar to the equivalent region in *S. cerevisiae* than was previously believed. In addition, the new estimate shows further similarities with respect to the position of known processing sites as well as the binding of the U3 snoRNA.

Based on the structural analysis results presented in chapter four, a series of 17 substitution mutations was created in order to more specifically determine which areas of each of the helices were essential for 18S rRNA maturation. The results presented as chapter five show that the sequence and probably the structure at the A0' site is critical for 18S rRNA maturation. Also, it was shown that the sequence integrity at the end of helix three is not of critical importance, but maintenance of the secondary structure in this area is. Finally, although there are seven bases conserved in *S. pombe* and *S.cerevisiae,* this study revealed that either not all of these bases are important for U3 snoRNA binding in *S. pombe,* or the 3' GAG sequence is critical for processing for an reason unrelated to the action of the U3 snoRNP. Whichever the case further studies in both organisms are needed to determine which of these possibilities exist. Collectively all of these results show that processing of the 5' ETS is more dependent on structural features in this spacer than previously believed and it would appear that the cell has evolved a variety of sequences/structural features to ensure that these sequences are removed correctly.

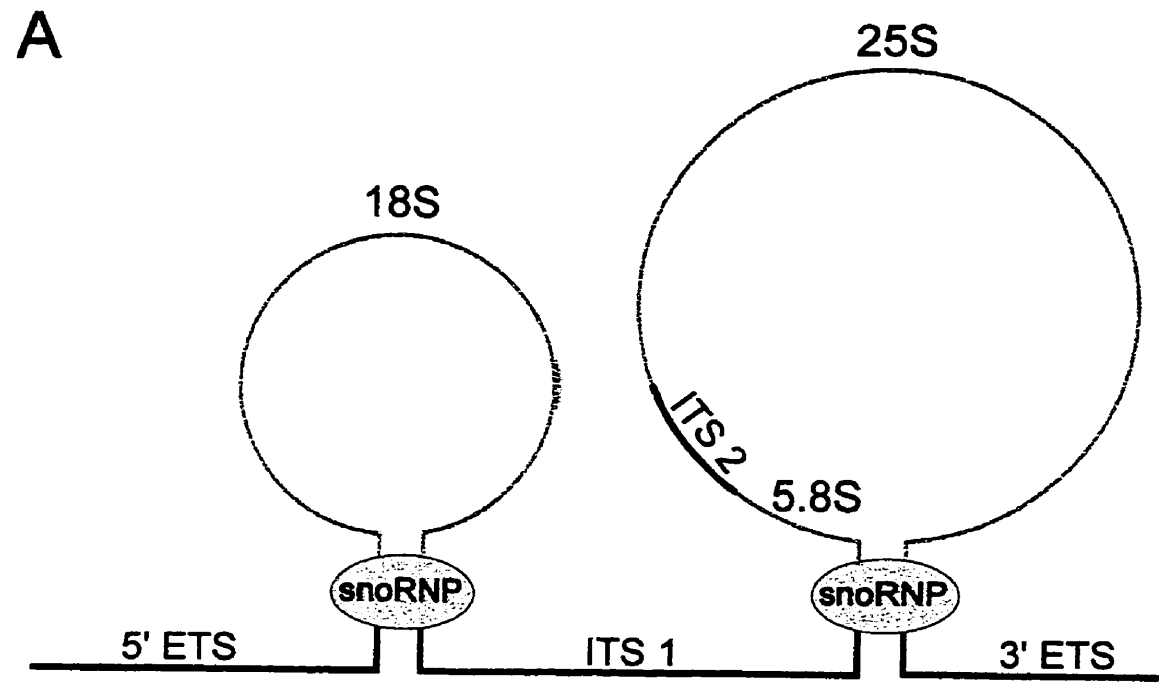
Several lines of evidence from our lab have led to the proposal that the transcribed spacers and elaborate processing mechanisms used to remove them may be acting as a quality control mechanism which helps to insure the quality of the RNA components that are incorporated into ribosomes. The data generated from the mutagenesis studies in this thesis have provided additional support for this hypothesis. In all cases, where mutation in the 5' ETS of *S. pombe* caused a decrease in the amount of 18S rRNA observed, although not completely proportional there was an accompanying decrease in the maturation of the 5.8S rRNA. These data suggest that processing of these distant regions of the pre-rRNA

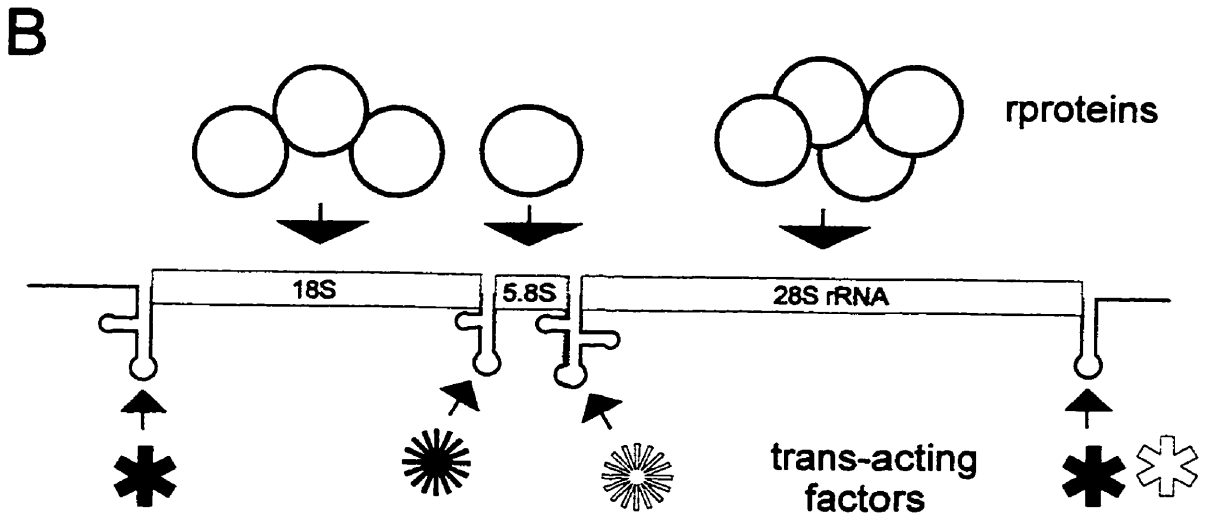
molecule are interdependent and may be dependent on a common processing domain. This proposal of a common processing complex involving all of the transcribed spacers is not consistent with models for the independent processing of the SSU and LSU rRNAs (van Nues et al. 1995, Morrissey and Tollervey, 1995) (Fig 6A). The previous models were proposed in part to explain evidence for cis-acting elements, trans-acting factors and ribosomal proteins which were found to influence mainly the maturation of either the LSU or SSU rRNAs. However, these models cannot explain the increasing amount of evidence for a processing interdependence of the SSU and LSU rRNAs. For example, deletion of ITS2 can affect processing of the whole transcript and reduce the levels of mature 18S rRNA to approximately ten percent (Good et al. 1997a). Deletions in ITS1 can completely inhibit 18S rRNA maturation and deletions which affect 3' ETS processing can reduce the amount of the SSU rRNA to approximately 50 % (unpublished data). Finally, in every case where mutation in the 5' ETS abolished production of 18S rRNA, there was also a decrease in the amount of the LSU rRNAs observed. These results are consistent with a model in which the distant elements interact to form a common complex eg. Fig. 6B, and abnormal transcripts which are not properly integrated into such a complex are completely or partially degraded. While this role of the spacers may seem wasteful in terms of cell energenics, the loss of energy associated with synthesis and degradation of the pre-rRNA might be negligible if they prevent the formation of dysfunctional ribosomes. Quality control during ribosome biogenesis may be the cell's only opportunity to control the ribosome's quality as it is unlikely that their integrity can be tested once formed. Therefore, in view of the results and the potential harm that could arise from dysfunctional ribosomes, it is suggested

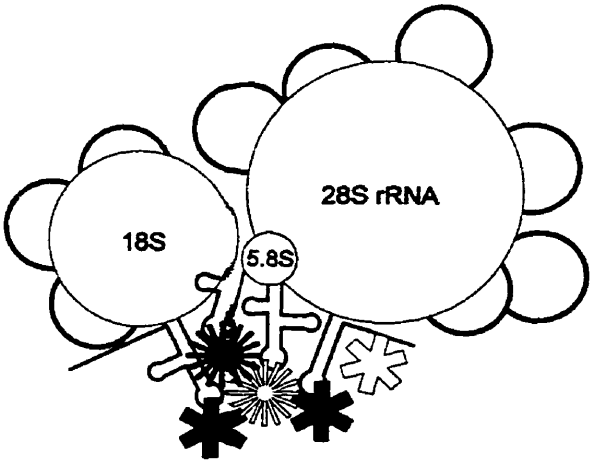
Figure 6.1 rRNA maturation schemes

A. Previously proposed split maturation scheme. Based on results from *S. cerevisiae* a split maturation scheme for rRNA processing was proposed. In this model, processing of the 18S rRNA is independent of the LSU rRNAs. The mature rRNAs as well as the spacer sequences are indicated. SnoRNPs thought to be involved in the processing reactions are also shown. This model was adapted from Morrissey and Tollervey (1995).

B. rRNA processing in a common domain. Efficient processing of the pre-rRNA requires ribosomal proteins, as well as many transacting factors to assemble with the spacer sequences in a common complex (domain).


A
25S
18S
ITS 2
5.8S
snoRNP
snoRNP
5' ETS
ITS 1
3' ETS


B
rproteins
18S
5.8S
28S rRNA
trans-acting
factors


28S rRNA
18S
5.8S

that normal rRNA processing requires that the rRNA spacers interact correctly with the other rRNA sequences and protein components in the pre-ribosomal particle. Otherwise, normal maturation is halted and the transcripts are degraded. In any event, if this is the case, this role for the rRNA spacers does not exclude other functions for the transcribed spacer sequences in transcription and ribosome formation.

While the work presented here has identified additional features in the 5' ETS that are important for pre-rRNA processing, it has also revealed new areas that need further research. For example in the present study the bases encompassing the A0' processing site were shown to be essential for rRNA maturation. Similar studies should be conducted in order to determine if the other processing sites which was previously identified in the 5' ETS (Good et al., 1997b) are also essential. Although the U3 snoRNP was shown to be essential in several organisms the binding site in *S. pombe* has yet to be identified unequivocally. Seven nucleotides of the U3 snoRNA binding site from *S. cerevisiae* were shown in this study to be conserved in the same relative position in the *S. pombe* 5' ETS and three of these bases (GAG) were essential for processing. Further studies need to be conducted in this area to determine if this site does indeed represent the U3 snoRNP binding site and future parallel studies in both organisms should determine if the GAG sequence identified here is the essential contact point in the 5' ETS. The initial mutagenic survey performed in this work led to the hypothesis that the sequences in the 5' end of the 5' ETS may be necessary for processome complex formation. Work has been initiated based on these mutations and gel mobility shift assays to determine if this is correct. Finally to date systems are not available in higher eukaryotes to investigate whether pre-rRNA processing occurs by a split maturation

scheme (as seen in *S. cerevisiae*) or is performed in a common domain as the studies performed here have shown. Unfortunately until such systems are available it will remain unknown which of these schemes are most common. Hopefully future developments of such systems will provide an answer to this question.

In conclusion, the results presented in this thesis provide new information on the structure and removal of the 5' ETS sequences in the pre-rRNA of *S. pombe*. The mutational analyses show that there are many structural elements within this spacer that affect its removal and the maturation of the 18S rRNA is far more dependent on the 5' ETS than was previously believed. In addition, this work has provided further strong support for the hypothesis that the spacer sequences have evolved, at least in part, as part of a quality control mechanism for ribosome biogenesis.

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