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Mutations affecting excision of the intron from a eukaryotic dimeric tRNA precursor

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The nucleotide sequences of a *Schizosaccharomyces pombe* opal suppressor serine tRNA gene (*sup9-e*) and of 12 *in vivo*-generated mutant genes, which have lost the ability to suppress UGA mutations, have been determined. Analysis of the expression of these genes in *Saccharomyces cerevisiae in vitro* and *in vivo* systems has revealed defects in tRNA gene transcription and precursor tRNA processing. Single base changes in the D-loop, the intron and the extra arm affect the efficiency of splicing of the tRNA precursors while an anticodon stem mutation may affect the accuracy of this process. Two mutations which occur in the intervening sequence of the *sup9-e* gene allow an alternate tRNA base pairing configuration. Transcription of the *sup9-e* gene and of the adjacent $tRNA^{Met}$ gene (located 7 bp downstream) is essentially abolished *in vivo* by a G→A19 mutation in the $tRNA^{Ser}$ gene, suggesting that $tRNA^{Met}$ may be derived solely via processing of the $tRNA^{Ser}$-$tRNA^{Met}$ dimeric precursor.

Key words: Schizosaccharomyces pombe/suppressor inactive mutants/tRNA processing/tRNA gene transcription

Introduction

Significant advances in our understanding of tRNA structure-function relationships and of the mechanism of protein biosynthesis have been achieved through the study of nonsense suppression (McClain, 1977; Steege and Söll, 1979; Ozeki *et al.*, 1980; Piper, 1980; Kohli *et al.*, 1980; Sherman, 1982). The analysis of the expression of suppressor tRNA genes containing inactivating second-site mutations has been invaluable in this respect. Initially, studies involving second-site mutations were carried out using tRNAs from *Escherichia coli* and bacteriophage T4. These have yielded a wealth of information concerning tRNA processing, nucleotide modification and aminoacylation (McClain, 1977; Steege and Söll, 1979; Ozeki *et al.*, 1980). In eukaryotes, similar studies have centered on the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. Collections of point mutations within suppressor tRNA genes which result in the loss of suppressor activity have been generated spontaneously (Kurjan *et al.*, 1980; Allison *et al.*, 1983) and by chemical mutagenesis (Pearson, 1983). Complementing this work, *in vitro* methods have been employed to introduce point mutations into non-suppressor tRNA genes (Folk and Hofstetter, 1983; Traboni *et al.*, 1984). When tested in a variety of *in vitro* and *in vivo* systems many of the mutant genes have been found to have altered transcriptional efficiencies and/or defects in the processing of the resulting precursor tRNAs (Allison *et al.*, 1983; Pearson, 1983; Folk and Hofstetter, 1983; Traboni *et al.*, 1984; Koski *et al.*, 1980, 1982; Nishikura *et al.*, 1982; Hopper and Kurjan, 1981). Mutations which affect template activity or the ability of the gene to compete *in vitro* against a reference tRNA gene for limiting transcription factors are clustered in regions of conserved nucleotide sequence (Allison *et al.*, 1983; Pearson, 1983; Folk and Hofstetter, 1983; Traboni *et al.*, 1984), and with a few exceptions, are within the D- and T-transcription control regions (nucleotides 8–21 and 50–64, respectively) as defined by gene deletion experiments (Galli *et al.*, 1981; Sharp *et al.*, 1981; Schaack *et al.*, 1984). In addition to these regions an 'extra loop promoter element' has been postulated based on the ability of nucleotides 45, 46 and 48 (in genes coding for tRNAs with small extra loops) to affect transcriptional efficiency (Allison *et al.*, 1983; Traboni *et al.*, 1984; Ciampi *et al.*, 1982; Ciliberto *et al.*, 1982). The extent of this region and its relationship to either of the other control regions remains unknown however, as does the existence of equivalent nucleotides in genes coding for tRNAs with large extra loops.

A number of single base changes within eukaryotic tRNA genes have been identified which affect processing of the precursor tRNAs (Folk and Hofstetter, 1983; Traboni *et al.* 1984; Nishikura *et al.*, 1982). In the most detailed study, the effects of 16 point mutations in the *SUP4-0* $tRNA^{Tyr}$ gene were examined by microinjection into *Xenopus* oocytes (Nishikura *et al.*, 1982). The conclusions reached from this work are in general agreement with those from earlier studies on tRNA maturation in prokaryotes (Altman, 1978; Mazzara and McClain, 1980): the processing enzymes appear to recognize the overall tRNA conformation. Mutations which block a particular processing step invariably alter the secondary or tertiary structure of the precursor tRNA. In eukaryotes, this concept is likely to also be applicable to tRNA splicing (Peebles *et al.*, 1983), although mutations in the tRNA which affect this process have been found primarily in the anticodon stem and loop region (Nishikura *et al.*, 1982; Colby *et al.*, 1981; Mattoccia *et al.*, 1983).

In the course of studies on informational suppression in *S. pombe* two efficient serine-inserting UGA suppressors were characterized (Kohli *et al.*, 1977, 1979, 1980; Hofer *et al.*, 1979). The genes were designated *sup3-e* and *sup9-e*. The *sup3-e* tRNA and its gene have since been isolated and sequenced (Rafalski *et al.*, 1979; Hottinger *et al.*, 1982). These studies showed: (i) that the *sup3-e* gene was generated by a TGA→TCA anticodon mutation in a $tRNA^{Ser}_{UCA}$ gene and (ii) that the *sup3-e* gene shared an unusual gene arrangement with the *S. pombe sup12*$^+$ $tRNA^{Ser}_{UCG}$ gene. Both genes are flanked to the 3′ side by a completely conserved DNA sequence comprising a 7-bp spacer and an initiator $tRNA^{Met}$ gene (Hottinger *et al.*, 1982; Mao *et al.* , 1980). In addition, each locus has been shown to direct the synthesis of a dimeric tRNA precursor *in vitro* (Pearson, 1983; Mao *et al.*, 1980).

Genetic evidence supports the view that *sup9-e* in its wild-

type form (*sup9*$^+$) may also code for a minor tRNA$^{Ser}_{UCA}$ species (Munz *et al.*, 1981). To confirm these data and to extend our analysis of the expression of *S. pombe* serine tRNA genes and their mutants (Hofer *et al.*, 1979) we undertook the isolation of the *sup9-e* gene. The clone we obtained was subsequently used in the isolation of wild-type and second-site alleles of *sup9*. We report here the sequence of these genes and the *in vitro* and *in vivo* characterization of their ability to be transcribed and processed.

Results

Isolation of the sup9$^+$ *serine tRNA gene and mutants derived from it*

Since *S. pombe* suppressor tRNA genes have been shown to be active in *S. cerevisiae* (Hottinger *et al.*, 1982, 1984) we attempted to isolate the *sup9-e* gene by transforming an *S. cerevisiae* UGA mutant strain with DNA from an *S. pombe* *sup9-e* strain and selecting for gene function. *S. pombe* DNA ligated into the plasmid vector YRp17 was transformed directly into the *S. cerevisiae* multiple auxotroph *his4-260*(UGA) *leu2-2*(UGA) *trp1-1*. Transformants were selected initially for the presence of the plasmid (TRP$^+$ phenotype) and subsequently for suppressor activity (prototrophy). Cummins and Culbertson (1981) have noted that large numbers of spontaneously-generated endogenous suppressors may be recovered using the direct transformation procedure. We therefore sought to minimize the appearance of these colonies by limiting the supply of nutrients (see Materials and methods). Despite this measure the majority of the transformants analyzed displayed a stable HIS$^+$ LEU$^+$ phenotype. Only seven transformants were found which showed concomitant instability of all three markers. A plasmid capable of re-transforming the *S. cerevisiae* UGA mutant strain to prototrophy was recovered from one of these transformants. A restriction map of the 3-kb *Eco*RI insert carried on this plasmid is shown in Figure 1A. Subcloning and re-transformation showed that the supressor was contained on a 1.55 kb *Cla*I/*Eco*RI fragment. Subsequently, nucleotide sequencing studies located the *sup9-e* gene ~60 bp from a unique *Hpa*I site (Figure 1A). The *sup9-e* tRNA sequence inferred from the gene sequence is shown in Figure 2. This sequence, including the 15 nucleotide intron, differs from the corresponding *sup3-e* sequence by only a U→C change at the tip of the extra arm of the tRNA. Analysis of the sequence downstream from the *sup9-e* gene revealed the same 7-bp spacer and initiator tRNA^{Met} gene as seen at the *sup3* and *sup12* loci (Hottinger *et al.*, 1982; Mao *et al.*, 1980).

Twenty-one nitrosoguanidine-induced suppressor inactive revertants mapping to 10 sites at the *sup9-e* locus have previously been characterized (Hofer *et al.*, 1979). Using the positive selection vector pTR262 and a *sup9-e* restriction fragment as a probe, we isolated the *sup9* gene from each of these mutant strains as well as from a strain carrying the wild-type allele, *sup9*$^+$. In each case the gene was recovered on a 5.3-kb *Hind*III fragment. A restriction map of this insert is presented in Figure 1B. The results of nucleotide sequence analysis of the wild-type and mutant genes are shown in Table I and Figure 2. The *sup9-e* suppressor, like *sup3-e* (Hottinger *et al.*, 1982) is generated by a G→C change in the anticodon of a tRNA$^{Ser}_{UCA}$ gene. The *sup3*$^+$ and *sup9*$^+$ genes, therefore, encode redundant tRNAs. Among the 21 independently isolated second-site revertants, 12 different mutations in the *sup9-e* gene were found. Ten of these are located in the

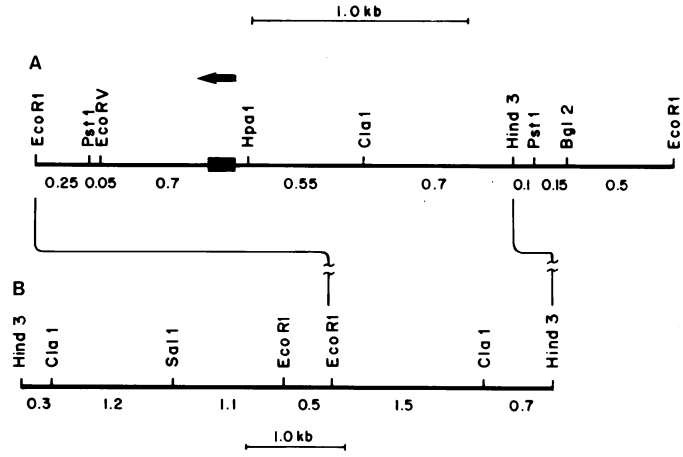

Fig. 1. Restriction maps of two clones containing the *S. pombe sup9* gene. **(A)** Shows the restriction map of a 3-kb *Eco*RI insert from a YRp17 clone containing the *sup9-e* gene. The location of the gene (box) and the transcription direction (arrow) were determined by DNA sequence analysis (Pearson, 1983). **(B)** Shows the restriction map of a 5.3-kb *Hind*III insert from a pTR262 clone containing the *sup9* gene.

mature tRNA coding sequence. The remaining two mutations occur in the intervening sequence. In contrast to the only other example of a suppressor-inactivating intervening sequence mutation (Koski *et al.*, 1982), neither of these mutations creates an RNA polymerase III termination signal. The physical location and the genetic map position of these mutants (Table I) differ slightly. In general, the two maps are co-linear, although inconsistencies involving sites 7–10 (Table I) are apparent. They were anticipated earlier (Hofer *et al.*, 1979) and may now be interpreted as being the result of weak marker specific effects at two sites. The revertants mapping to site 7 (with the exception of *r131*) and those mapping to site 9 display reduced recombination frequencies in hetero-allelic crosses when compared with those mapping at other sites including sites 8 and 10.

The in vitro processing of sup9$^+$ *and sup9-e transcripts is different*

To determine whether any of the second-site mutations affect *sup9* tRNA biosynthesis, plasmid DNAs containing *sup9*$^+$, *sup9-e* and each of the mutant suppressor genes were transcribed in an *S. cerevisiae* cell-free extract. This extract can specifically transcribe RNA polymerase III genes and carry out nucleolytic processing of the *in vitro* synthesized transcripts (Klekamp and Weil, 1982). The results are presented in Figure 3. As was the case for *sup3* and *sup12* (Pearson, 1983; Mao *et al.*, 1980), the arrangement of the *sup9* gene in tandem with an initiator tRNA^{Met} gene leads to the synthesis of a dimeric tRNA precursor.

A comparison of the products obtained in transcriptions of *sup9*$^+$ and *sup9-e* reveals some striking differences. These include: (i) the apparent accumulation of radioactivity in two RNAs which migrate between the dimeric tRNA precursors and the tRNA^{Ser} plus intervening sequence species: (ii) the absence of a detectable intron-containing tRNA^{Ser} species; (iii) multiple bands at the position corresponding to mature tRNA^{Ser}; (iv) multiple bands and alterations in the sizes of the tRNA^{Ser} half molecules. Pulse-chase experiments have shown (Pearson, 1983) that radioactivity incorporated into the dimeric tRNA precursors of *sup9*$^+$ and *sup9-e* may be followed into all of the other major RNA transcripts observ-

Table I. Characteristics of *SUP9-e* second-site mutations

Mutation	Revertant notation[a]	Revertant site in fine structure map[b]	Base change	Effect on tRNA structure	Suppression in an *S. cerevisiae* UGA mutant strain[c]
G14	r104	1	A→G	Changes invariant A	–
A19	r101	2	G→A	Changes invariant G	–
A30	r85	3	G→A	Changes highly conserved G and abolishes base pair	–
CAU37:4	r192[d] r202[d]	4	UG→CAU	Alternate base paired configuration	+
A37:13	r4[e] r5[e]	5	G→A	Alternate base paired configuration	–
C39	r14	6	U→C	Abolishes base pair	–
A46	r23 r64	8	G→A	Abolishes base pair	–
U47:7	r131	7	C→U	Base pair weakened	+
Ai47:8	r46 r214	7	A insertion		±
Ai48	r89 r133 r137 r176	7	A insertion		–
A53	r49	10	G→A	Changes invariant G and abolishes base pair	–
A68	r68 r77 r102	9	G→A	Abolishes base pair	–

The mutations are listed in the 5′→3′ direction and have been named according to the system of Sprinzl *et al.* (1980).
[a]Revertant notation is as described by Hofer *et al.* (1979).
[b]The relative location of revertants in the *sup9-e* fine structure map (Hofer *et al.*, 1979) is represented numerically. Sites 1 and 10 correspond to the rightward and leftward boundaries, respectively.
[c]The ability of the mutant *S. pombe* genes to suppress *S. cerevisiae* UGA mutations was examined by transforming strain YH-D5 as described in Materials and methods. From overnight cultures in TRP^+ selection medium ~10^4 cells of each transformant were spotted on minimal medium agar plates. Growth was monitored at 30°C for 5 days. + denotes suppression, ± weak suppression and – no suppression.
[d]Osmotic remedial conditional allele (Hofer *et al.*, 1979).
[e]Temperature-sensitive conditional allele (Hofer *et al.*, 1979).

ed. Thus, the above differences and similar variations seen in transcriptions of the second-site mutants must occur at the level of RNA processing. Since the *sup9-e* precursor tRNA differs from the $sup9^+$ RNA by the loss of one G-C base pair in the anticodon/intervening sequence region of potential complementarity (Figure 2), a change in the conformation of the tRNA precursors, analogous to that proposed for *SUP4-0* $tRNA^{Tyr}$ (Nishikura *et al.*, 1982), may explain the different processing pattern.

In regard to the aberrant $tRNA^{Ser}$ half molecules, RNA sequence data indicate that at least two cuts are necessary to generate these species as most nucleotides originating from the intron are absent (unpublished data). However, it is not clear whether the splicing endonuclease or another enzyme is involved in their formation.

In vitro analysis of the mutant tRNA genes

When the mutant *sup9* genes were transcribed in the *S. cerevisiae* extract differences in the amount of transcripts and in the nature of the processing products were observed (Figure 3).

Four mutants (G14, U47:7, A46 and A37:13) appear to affect the splicing endonuclease activity. In comparison to *sup9-e*, they produce increasing (in the order given) amounts of an RNA corresponding to the intron-containing $tRNA^{Ser}$ precursor (Figure 3). This suggests that the precursor tRNAs of these mutants are spliced less efficiently. An examination of the sequence of the A37:13 mutant reveals that this single base change creates the sequence UCUAA (Figure 2) which is a direct repeat of the sequence at the 3′ side of the anticodon stem. This may lead to base pairing between the 5′ side of the anticodon stem and the complementary region of the intron. This alternate tRNA precursor structure may not be easily cleaved by the splicing endonuclease. It is interesting to note that the *S. pombe* strains which contain the A37:13 mutation display a temperature-sensitive suppressor phenotype (see Table I). Residual suppression is observed at 20°C but not at higher temperatures suggesting that the alternative conformer is more stable than the one shown in Figure 2.

Base changes in three mutants (A19, Ai48 and A53) lead to a reduction in their transcriptional efficiency. An effect on transcription (decreased template activity or competitive ability) of mutations in positions 19 and 53 has previously been seen in other eukaryotic tRNA genes (Allison *et al.*, 1983; Pearson, 1983; Folk and Hofstetter, 1983; Traboni *et al.*, 1984) although this is the first account of an A at position 53. The Ai48 mutation is of interest since it poses the question: does the transcription machinery recognize the inserted base as being located at position 48 or 49 in the tRNA gene? Evidence sugesting that the inserted A displaces the C residue

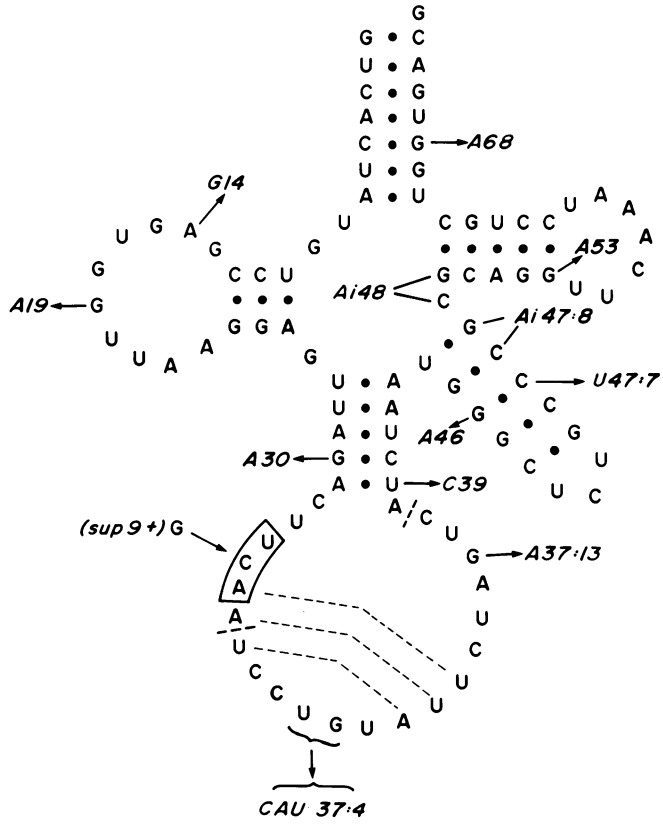

Fig. 2. A cloverleaf structure representation of the intron-containing *sup9-e* tRNA precursor. The RNA sequence is deduced from the gene sequence. The location and base change for each second-site mutant is indicated. The anticodon is boxed and the splice sites are shown by dashed lines. Potential base pairing between the anticodon region and the intron is also shown.

at position 48 is as follows: a C residue at position 48 in tRNA genes and at an analogous position in non-tRNA genes transcribed by RNA polymerase III is highly conserved (Allison *et al.*, 1983); there is no consensus base at position 49; and a C→T transition at position 48 has been shown to affect transcriptional efficiency whereas no such effect has been observed for base substitutions at position 49 (Allison *et al.*, 1983; Pearson, 1983; Traboni *et al.*, 1984).

As discussed above, the *in vitro* system produces aberrant $tRNA^{Ser}$ half molecules for *sup9-e* and most of the mutant suppressor genes. Three mutations cause changes in the mobility of these RNA species. The opposing extra arm mutations, A46 and U47:7, produce the same sized aberrant tRNA half molecules (apparently 1–2 nucleotides larger than the corresponding *sup9-e* species). However, when electrophoresed on 30% formamide-8 M urea gels, these species co-migrate with those of *sup9-e* (data not shown). This result leads us to conclude that the change in mobility seen in Figure 3 probably results from the relief of band compression (Figure 2). The C39 mutation causes a 1–2 nucleotide decrease in the size of the same products (compared with *sup9-e*). The size differences for this mutant are less obvious in Figure 3 than in other gels (not shown) owing to a salt effect in this lane at the bottom of the gel.

The pattern of products obtained for A30, CAU37:4, Ai47:8 and A68 was not appreciably different from the *sup9-e* control. The possibility that these mutations may influence one or more processing steps remains (cf. *in vivo* data below), since such effects may be masked by the alteration in the *in vitro* processing pattern which accompanies the formation of the suppressor anticodon.

In vivo expression of the mutant sup9 genes

To examine transcription and processing *in vivo*, RNA from *S. cerevisiae* transformants harboring the various *sup9* genes was subjected to Northern hybridization analysis. Specific detection of the *S. pombe* gene products was achieved using a *sup9-e* fragment (*ClaI/Eco*RV, Figure 1) containing the $tRNA^{Ser}$-$tRNA^{Met}$ genes. As expected from the known sequences of the $tRNA^{Ser}$ and $tRNA^{Met}$ species in the two yeasts, little cross-hybridization between the probe and *S. cerevisiae* RNA occurs (Figure 4). The pattern of hybridization obtained with RNA from a $sup9^+$ transformant correlates with the *in vitro* transcription pattern of this gene (compare Figures 3 and 4). A comparison between the hybridization patterns for $sup9^+$ and *sup9-e* reveals that significantly less hybridizable RNA is present in *sup9-e*-transformed cells. This feature is observed consistently for different preparations of RNA from different isolates. Another conserved feature of these transformed cells is their growth rate. In media where only the presence of the recombinant plasmid is required for growth, the doubling times for cells carrying $sup9^+$ or the mutant *sup9-e* genes is between 230–330 min, as opposed to 370–450 min for cells carrying the active suppressor. The lowered growth rates of suppressor-containing strains is a well-known phenomenon and is probably due to the deleterious nature of the suppressor tRNA on the chain termination process (Hottinger *et al.*, 1982). In addition, it is conceivable from the hybridization data (Figure 4) that the presence of the suppressor in the cell may lead to a reduction in plasmid copy number and/or in the rate of RNA polymerase III transcription. The possibility that the reduction in hybridization to *sup9-e* transformant RNAs may be due to an increase in the rate of degradation or processing of *sup9-e* transcripts is unlikely, since RNA from second-site transformants contain amounts of the dimeric tRNA precursor species and $tRNA^{Met}$ similar to those seen for $sup9^+$.

Mutations which affect transcription *in vitro* (A19, Ai48 and A53) show a similar effect *in vivo*. In particular, transcripts from the mutant A19 are barely detectable. The drastic effect of this mutation in the $tRNA^{Ser}$ gene on the amount of $tRNA^{Met}$ synthesized has some interesting implications. Firstly, it provides direct evidence that for the *S. pombe* $tRNA^{Ser}$-$tRNA^{Met}$ genes, $tRNA^{Met}$ is derived via processing of the dimeric RNA precursor and not initiated independently by the internal $tRNA^{Met}$ gene promoters. Possibly the $tRNA^{Ser}$ gene provides an inhibitory 5′-flanking sequence for this gene (Dingermann *et al.*, 1982). Secondly, it provides a useful internal standard for tRNA stability. The amount of hybridization to $tRNA^{Ser}$-specific species should be equivalent to the amount for $tRNA^{Met}$ (see Figure 4, $sup9^+$ and *sup9-e*). From this it is clear that many of the second-site mutations in the *sup9-e* gene produce $tRNA^{Ser}$ species which have a reduced half-life. The appearance of mutant $tRNA^{Ser}$-specific species nevertheless provides an indication of the ability of the processing enzymes to act on these substrates since the hybridization intensities reflect steady state concentrations of these RNAs.

Several mutations affect tRNA splicing *in vivo*. Mutant A37:13, was found to accumulate the intron-containing

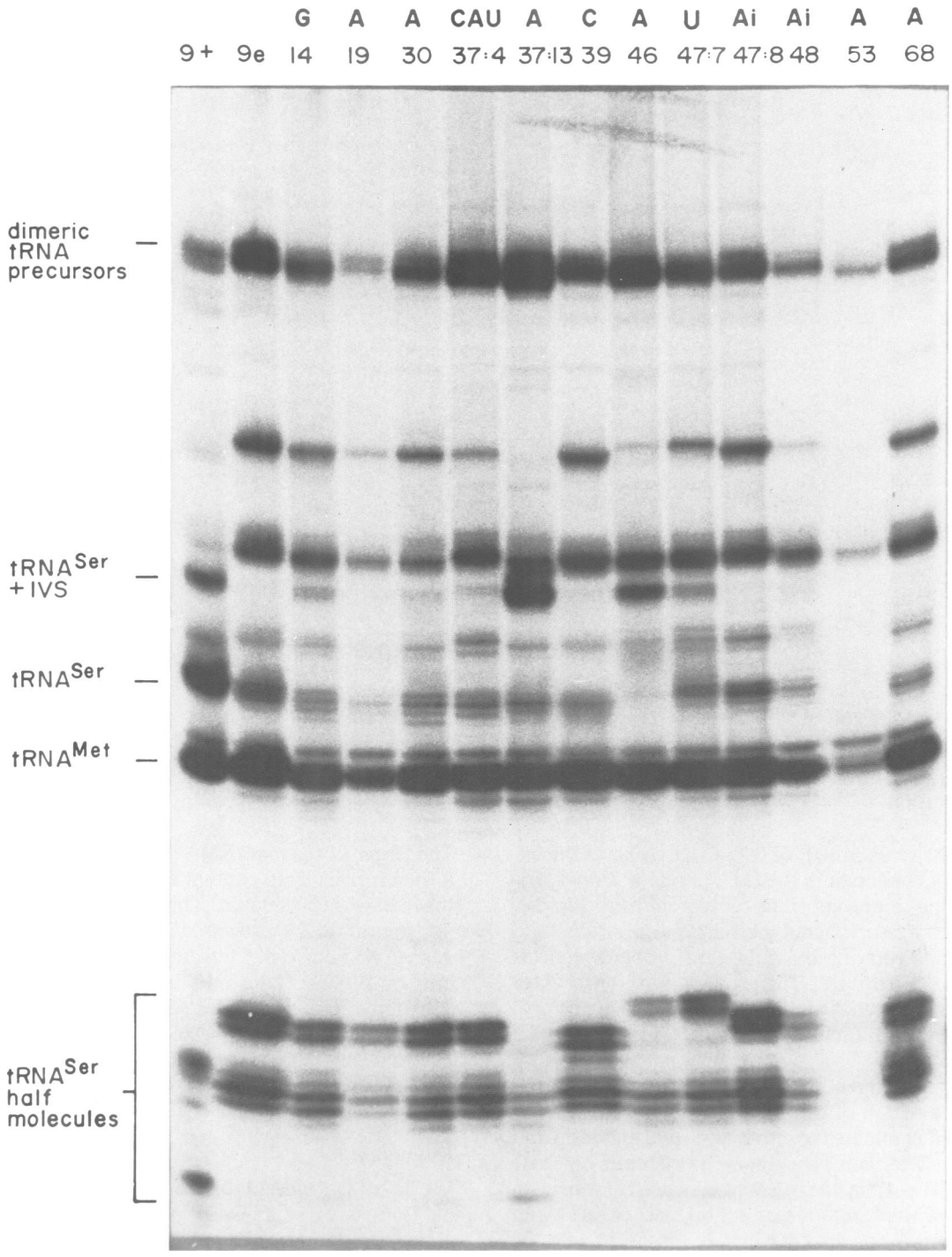

Fig. 3. *In vitro* transcription of *sup9* wild-type, suppressor and second-site alleles. After recloning the *ClaI/EcoRI* fragment (Figure 1) from each *sup9* gene into YIp5, plasmid DNAs were prepared and used to direct transcription in an *S. cerevisiae* cell-free extract. Transcription reactions were carried out using saturating amounts of the template DNAs under conditions such that the processing of the *sup9*$^+$ dimeric precursors into tRNA$^{Ser}_{UCA}$ and tRNA^{Met} was almost complete. All the products observed are insert-specific with the exception of the band between tRNA^{Ser} and tRNA^{Met} which appears in the absence of exogenous DNA. The products indicated by arrows were identified by RNase T1 fingerprinting and nearest neighbor analysis (D. Frendewey and I. Willis, unpublished data).

tRNA^{Ser} species *in vitro* (Figure 3). A corresponding band is also seen in the *in vivo* analysis (Figure 4). Similarly, the accumulation of the presumptive splicing substrate in RNA from G14 and Ai47:8 transformants suggests that these alterations affect the efficiency of splicing *in vivo*. For mutants C39, A46 and U47:7, hybridization to tRNA^{Ser}-specific precursors is observed, but these species do not appear to accumulate. This may be due to the instability of the precursor tRNAs. For C39, an alternative explanation which takes into account the *in vitro* observations for this mutant (Figure 3), might be that aberrant tRNA^{Ser} half molecules are produced which cannot be ligated. Whether these RNA species accumulate *in vivo* is not known. In the case of mutant U47:7, a small amount of mature tRNA^{Ser} is produced as shown by Northern analysis (Figure 4) and by the ability of this mutant to suppress UGA mutations in *S. cerevisiae* (Table

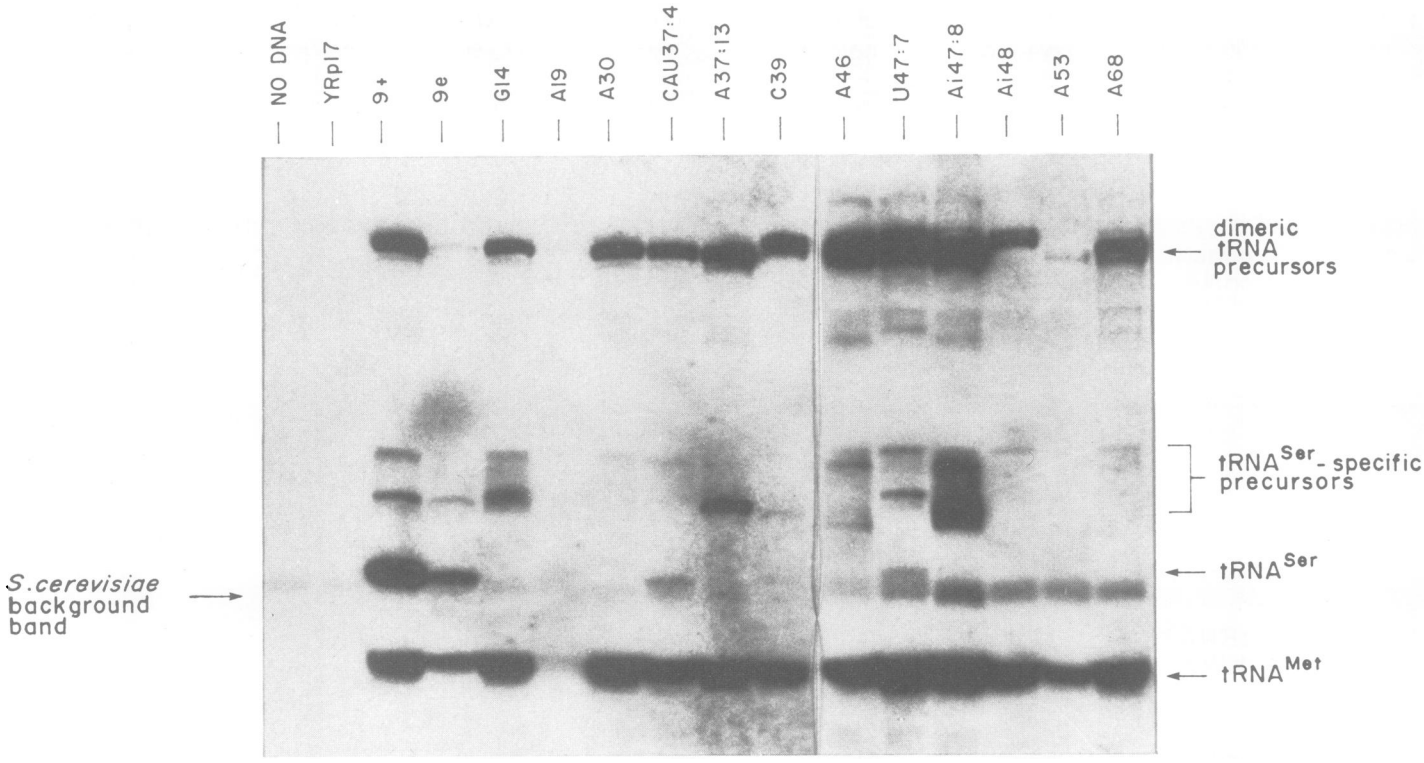

Fig. 4. Northern hybridization analysis of RNA from *S. cerevisiae* transformants. Equal amounts of RNA from *S. cerevisiae* transformants harboring each *sup9* gene (recloned into YRp17) were electrophoresed on a 10% polyacrylamide gel. Electrophoresis conditions were not fully denaturing and therefore mobilities may be altered by secondary structure effects. After transfer of the RNA to DMB-paper, hybridization was carried out using the *sup9-e* $tRNA^{Ser}$-$tRNA^{Met}$ dimeric gene as a probe. The identity of the hybridizing species was determined on the basis of the hybridization pattern obtained using $tRNA^{Ser}$- and $tRNA^{Met}$-specific probes (I. Willis, unpublished data).

I). Presumably the replacement of a G-C base pair in the extra arm with a G-U base pair in the U47:7 mutant is not sufficiently destabilizing to prevent some correct splicing. The difference in the suppressor phenotype between *S. pombe* and *S. cerevisiae* strains carrying this mutation probably reflects the difference in gene dosage. This explanation is also likely to apply to mutants CAU37:4 and Ai47:8, both of which produce small amounts of functional $tRNA^{Ser}$ in *S. cerevisiae* (Table I and Figure 4).

An analysis of the sequence created by the CAU37:4 mutation reveals that a region of complementarity exists, which if base-paired would eliminate the anticodon stem and loop and the extra arm. The complementary nucleotides include 37:2–37:7 and 41–47 in the *sup9-e* sequence as shown in Figure 2 and allow the formation of a 7-bp helix closed by an 11-nucleotide loop. A conclusion which is consistent with the *in vivo* hybridization pattern for this mutation might be that transcripts which adopt the alternative conformation are processed as far as the larger $tRNA^{Ser}$-specific precursor, and are unstable. $tRNA^{Ser}$ may be produced from transcripts which formed the normal structure, however, the frequency of this event is apparently low.

The sequence of processing events for the *S. pombe sup12* $tRNA^{Ser}_{UCG}$-$tRNA^{Met}$ precursor has been established in a *Xenopus* nuclear extract (Mao *et al.*, 1980). The first step in that scheme involved an RNase P-like cleavage to separate the two tRNAs. This was followed by maturation of the 5′ and 3′ ends of the $tRNA^{Ser}$ precursor prior to the removal of the intervening sequence. Based on the *in vitro* and *in vivo* data presented here, a similar sequence of processing steps occurs for the *sup9* $tRNA^{Ser}$-$tRNA^{Met}$ precursor in *S. cerevisiae*. The larger of the two $tRNA^{Ser}$-specific precursors in Figure 4 is therefore likely to contain flanking sequences in addition to the intervening sequence. This precursor is observed but does not appear to be processed further in the case of mutants A30, Ai48 and A68. Thus, these mutants may be defective in one or more of the end maturation steps (RNase P or 3′-trimming activity). This conclusion has been reached for a mutation analogous to A68 (Folk and Hofstetter, 1983). Alternatively, the instability of these mutant tRNAs may preclude the detection of further processing products.

Discussion

We have identified 12 different mutations among a collection of 21 'loss of suppression' mutants at the *sup9-e* locus of *S. pombe*. Many of these represent mutations at sites in the tRNA molecule of a type not previously described in eukaryotes. Upon *in vitro* and *in vivo* analysis in *S. cerevisiae* we have been able to relate the presence of the second-site mutations to particular defects in tRNA gene transcription and/or tRNA processing.

In vivo processing studies of mutant yeast tRNA genes in *Xenopus* oocytes (Nishikura *et al.*, 1982) have shown that the step most susceptible to alterations in the tRNA is end maturation (cleavage of the 5′ leader by RNase P, removal of the 3′ trailer and CCA addition). In contrast, our analysis of the *S. pombe sup9* alleles in *S. cerevisiae* indicates that removal of the intron may be more readily affected. Mutations in four different regions of the tRNA alter the efficiency and in some cases possibly the accuracy of this step (G14 in the D-loop, A37:13 in the intron, C39 in the anticodon stem

and A46, U47:7 and Ai47:8 in the extra arm). Each of these mutations disrupts the secondary or higher order structure of the tRNA and therefore provides direct evidence that the splicing endonuclease recognizes conserved structural features among its substrates. An indication of the sensitivity of this recognition is demonstrated by the G14 mutation which prevents the normal tertiary interaction between U8 and A14 (Rich, 1977). That this interaction may be of general importance for tRNA splicing in yeast is supported by the suggestion that the same mutation in the *SUP4-0* $tRNA^{Tyr}$ gene may affect tRNA processing (Allison *et al.* 1983). It is interesting to note that the absence of the U8-A14 interaction does not appear to affect significantly the activity of the *Xenopus* splicing endonuclease (Nishikura *et al.*, 1982). Differences in the structural requirements of the yeast and *Xenopus* enzymes regarding the anticodon/intervening sequence region have previously been suggested by Nishikura *et al.* (1982).

The analysis of three mutations in the extra arm of the *sup9-e* $tRNA^{Ser}$ suggests a 2-fold importance for this region in the splicing reaction. Firstly, base pairing within the large extra arm appears to be required for efficient splicing (A46 and U47:7 in Figure 3). Secondly, that splicing occurs at all, we suggest, is dependent on the ability of the long extra arm to fit conformationally with the yeast splicing endonuclease. Evidence for this comes from: (i) the analysis of the Ai47:8 mutation (Figure 4) which presumably alters the conformation of the extra arm relative to the rest of the tRNA and (ii) the knowledge that in order for mutations A46 and U47:7 to affect the efficiency of this reaction (Figure 3), the extra arm must be in close proximity to a part of the splicing endonuclease. This is clearly not an absolute requirement for splicing however, since not all intron-containing yeast tRNAs possess a long extra arm.

Another structural requirement for normal splicing is suggested by the C39 mutation which disrupts the last base pair in the anticodon stem and produces aberrant tRNA half molecules *in vitro* (Figure 3). The notion that these altered tRNA half molecules are cleavage products of the splicing endonuclease is supported by the observation that disruption of an equivalent base pair in an intron-containing proline tRNA results in the generation of a frameshift suppressor (M. Culbertson, personal communication). In apparent contrast to the C39 mutation in *sup9-e* (Figure 4), the proline tRNA half molecules must be ligated since the suppressor phenotype is expressed. Considering the conserved location of the intervening sequence in tRNAs (Peebles *et al.*, 1983), the juxtaposition of the splice sites relative to the last base pair in the anticodon stem may be important in determining which nucleotides are aligned in the active site of the splicing endonuclease. Alternatively, a 5-bp anticodon stem, alone, may be necessary for accurate splicing.

The location of the three transcription-down mutations (A19, Ai48 and A53) is in the expected regions of the tRNA gene (Allison *et al.*, 1983; Traboni *et al.*, 1984; Galli *et al.*, 1981; Sharp *et al.*, 1981; Schaak *et al.*, 1984; Ciampi *et al.*, 1982). The analysis of point mutations in several tRNA genes indicates that nucleotide position 48 but not 49 has a role in transcription promotion (Allison *et al.*, 1983; Pearson, 1983; Traboni *et al.*, 1984). The transcriptional effect of the Ai48 mutation may therefore be explained if the inserted A is recognized by the transcription machinery as being located at position 48. Implicit in this interpretation is a spatial relationship between the 'extra loop promoter element' and the 3′ internal transcription control region. Thus, although a comparison of large and small extra loop sequences among eukaryotic tRNAs reveals that C48 is the only highly conserved nucleotide in this region, a transcriptional equivalent of nucleotides 45 and 46 in tRNAs with small extra loops (Allison *et al.*, 1983; Ciampi *et al.*, 1982) may be predicted to reside 2–3 nucleotides 5′ to C48 in tRNAs with large extra loops. Evidence in support of this proposition is provided by DNase I protection experiments. The ability of proteins in a yeast nuclear or whole-cell extract to protect specifically the internal transcription control regions of several yeast tRNA genes has been reported by Klemenz *et al.* (1982) and Newman *et al.* (1983). Their data show that the 5′ boundary of the protected 3′ transcription control region is located ~4 nucleotides 5′ to C48 in genes coding for tRNAs with either small or large extra loops. An examination of the competitive ability of mutants U47:7 and Ai47:8 may be useful in determining the transcriptional effect of this region.

The ability to detect specifically *in vivo* synthesized products of the *sup9* dimeric gene together with the observation that $tRNA^{Met}$ is derived exclusively from the dimeric tRNA precursor (see A19 in Figure 4) has made possible qualitative determinations of the *in vivo* stability of the mutant $tRNA^{Ser}$ species. In general, it appears that mutations which disrupt the helical structure of the tRNA (A30, CAU37:4, A46, U47:7, A53 and A68) have a more pronounced effect on tRNA stability than those which alter only the tertiary or higher order structure (G14, A37:13 and Ai47:8). The mutation C39 has been omitted from this comparison although it appears to be consistent with the hypothesis. One interpretation of the *in vitro* and *in vivo* data for this mutant is that it may yield unligatable half molecules. Thus, the reduction in hybridization to $tRNA^{Ser}$ species may reflect an increase in the rate of splicing.

This work and other studies (Pearson, 1983) provide the basis upon which genetic and molecular biological approaches are currently being used to exploit the plasmid-mediated expression of *S. pombe* suppressor tRNA genes and their mutants in *S. cerevisiae* in an effort to define the cellular components involved in their biosynthesis.

Materials and methods

Isolation and sequence analysis of S. pombe sup9 alleles

DNA from the *S. pombe* strain *sup9-e ade1-40* was prepared (Hottinger *et al.*, 1982; Cryer *et al.*, 1975) using both Zymolyase 60 K and Novozym SP234 (Novo Laboratories, 3 ml of the 10 g/ml solution in 0.5 M citrate-phosphate buffer, pH 5.6 per 10 g wet weight of cells) to digest the cell wall. The inclusion of the Novozym which contains significant α-(1→3)-glucanase activity is essential for efficient spheroplasting of *S. pombe* cells. After digestion with *Eco*RI, eight independent ligations were performed. Each contained 12 μg of chromosomal *Eco*RI fragments, 8 μg of dephosphorylated, *Eco*RI-restricted YRp17 DNA and 0.2 Weiss units T4 DNA ligase in 250 μl. Ligated DNA was ethanol precipitated and dissolved in 10 mM Tris-HCl, 0.1 mM EDTA, pH 7.5 to a concentration of 1 μg/μl. This material was used to transform (Struhl *et al.*, 1979) directly the *S. cerevisiae* strain YH-D5 (Hottinger *et al.*, 1982). YH-D5 spheroplasts prepared from 100 ml cultures grown to a density of 2.2 x 10^7 cells/ml were transformed with each ligation reaction. Transformation reactions were divided into three tubes (0.33 ml/tube) each containing 1 ml of 0.5 x minimal medium overlay (Fink, 1970), 0.25% casamino acids (Difco) at 45°C. The tubes were incubated at 30°C for 1.5 days. The agar plugs containing thousands of microcolonies (10^3–10^4 cells/colony) were crushed and added to liquid minimal medium supplemented with subthreshold levels of histidine (0.01 mM) and leucine (0.1 mM). After incubation in a shaker water bath at 30°C for 4–6 days, cell material was spread on minimal medium agar plates. DNA prepared from transformants displaying

unstable TRP$^+$ HIS$^+$ LEU$^+$ phenotypes was purified by CsCl density gradient centrifugation, fractionated and used to transform *E. coli* strain HB101 to ampicillin resistance. Minilysates were made from *amp*$^+$ clones and the plasmid DNA analyzed by restriction endonuclease digestion and agarose gel electrophoresis. DNA prepared as described above from a wild-type *sup9* strain and 21 other *S. pombe* strains each carrying second-site mutations at the *sup9* locus was digested to completion with *Hind*III and cloned into the *Hind*III site of pTR262 (see Pearson, 1983). Transformants of *E. coli* strain BJ5183 were screened by colony hybridization (Pearson, 1983) using as probe the *Cla*I/*Eco*RV fragment of *sup-9e* (Figure 1). All *S. pombe* strains were obtained from Urs Leupold.

tRNA genes were sequenced by the 'dideoxy' method (Sanger *et al.*, 1977) with a *sup9* specific primer (GAGATAGCTTTCAAG). The primer is complementary to the tRNA coding strand and initiates synthesis at nucleotide −2 relative to the mature *sup9* tRNA sequence. tRNA genes were prepared by recloning the 1.55-kb *Cla*I/*Eco*RI fragment from each pTR262 clone into *Acc*I/*Eco*RI digested M13 mp8 (Messing and Vieira, 1982).

Subcloning and in vitro transcription of sup9 alleles

The procedure of Klekamp and Weil (1982) was used for the preparation of an RNA polymerase III transcription extract from *S. cerevisiae* and was kindly provided by Dr. J. Schaack. The construction of recombinant plasmid DNAs for *in vitro* transcription and for *in vivo* analysis involved recloning the 3.1-kb *Cla*I/*Sal*I fragment from each pTR262 clone (Figure 1B) into YRp17. Plasmid DNAs were prepared after transformation and amplification in *E. coli* HB101. The identification of an additional *S. pombe* tRNA gene within the 1.1-kb *Eco*RI/*Sal*I fragment (Figure 1B, H. Amstutz, personal communication) however, necessitated further recloning in order to simplify the *in vitro* transcription patterns. For this purpose the 1.55-kb *Cla*I/*Eco*RI fragment was recloned into YIp5 (Struhl *et al.*, 1979). Each 50 μl transcription reaction contained 10 mM Hepes, pH 7.9, 10 mM $MgCl_2$, 130 mM NaCl, 10% v/v glycerol, 0.5 mM DTT, 1.2 mM ATP, 0.6 mM each of CTP and UTP, 25 μM [α-^{32}P]GTP (8 Ci/mmol), 20 μg/ml supercoiled plasmid DNA and 5 μl of extract. Reactions were incubated at 25°C for 1 h. RNA was purified (Koski *et al.*, 1982) and resolved on a 6% polyacrylamide gel containing 8 M urea.

Northern hybridization analysis

YRp17 recombinants containing the various *sup9* alleles (see above) were transformed into *S. cerevisiae* strain YH-D5 (*his*$^-$ *leu*$^-$ *trp*$^-$). Colonies exhibiting instability of the *trp1* marker were grown for the preparation of RNA in minimal medium supplemented with histidine and leucine (600 ml cultures) to a density of 1.3 x 10^7 cells/ml. Low mol. wt. RNAs were prepared (Rubin, 1975) and resolved on 10% polyacrylamide gels containing 8 M urea (0.7 mm thick). Each lane contained 50 μg of RNA. Electrophoresis was at 5 mA and ambient temperature. Following equilibration of the gels in 200 mM sodium acetate, pH 4.0, the RNA was electrophoretically transferred to DBM-paper (240 mA for 4 h) using a BioRad Trans-Blot Cell (Hopper and Kurjan, 1981). Filters were hybridized in the buffer described by Alwine *et al.* (1977) (~75 μl/cm^2 DBM-paper) for 20 h at 42°C. Post-hybridization washes were at 65°C. The hybridization probe (~10^6 c.p.m./ml) consisted of the 1.3-kb *Cla*I/*Eco*RV fragment containing *sup9-e* (Figure 1A) and was labelled to a specific activity of 0.5 − 1.0 x 10^8 c.p.m./μg using T4 DNA polymerase and [α-^{32}P]dATP (3000 Ci/mmol).

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