Random mutagenesis of *Schizosaccharomyces pombe* SRP RNA: lethal and conditional lesions cluster in presumptive protein binding sites

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ABSTRACT

Signal recognition particle (SRP), a ribonucleoprotein composed of six polypeptides and one RNA subunit, serves as an adaptor between the cytoplasmic protein synthetic machinery and the translocation apparatus of the endoplasmic reticulum. To begin constructing a functional map of the 7SL RNA component of SRP, we extensively mutagenized the *Schizosaccharomyces pombe SRP7* gene. Phenotypes are reported for fifty-two mutant alleles derived from random point mutagenesis, seven alleles created by site-directed mutagenesis to introduce restriction sites into the *SRP7* gene, nine alleles designed to pinpoint conditional lesions, and three alleles with extra nucleotides inserted at position 84. Our data indicate that virtually all single nucleotide changes as well as many multiple substitutions in this highly structured RNA are phenotypically silent. Six lethal alleles and eleven which result in sensitivity to the combination of high temperature and elevated osmotic strength were identified. These mutations cluster in conserved regions which, in the mammalian RNA, are protected from nucleolytic agents by SRP proteins. The effects of mutations in the presumptive binding site for a fission yeast SRP 9/14 homolog indicate that both the identity of a conserved residue and the secondary structure within which it is embedded are functionally important. The phenotypes of mutations in Domain IV suggest particular residues as base-specific contacts for the fission yeast SRP54 protein. A single allele which confers temperature-sensitivity in the absence of osmotic perturbants was identified in this study; the growth properties of the mutant strain suggest that the encoded RNA is somewhat defective even at the permissive temperature, and is most likely unable to correctly assemble with SRP proteins at the non-permissive temperature.

INTRODUCTION

Signal recognition particle is a cytoplasmic ribonucleoprotein which targets ribosomes translating secretory proteins to the endoplasmic reticulum membrane (reviewed in 1). The extensively studied canine particle is composed of one RNA molecule (historically designated 7SL but now generally referred to as SRP RNA) and six polypeptides organized into two heterodimeric (SRP9/14 and SRP68/72) and two monomeric (SRP19 and SRP54) proteins (reviewed in 2). Using an *in vitro* translocation assay to assess function following biochemical manipulation of the particle, roles in the SRP cycle have been ascribed to all of the proteins except SRP19. A subparticle reconstituted in the absence of SRP9/14 is competent for translocation but does not exhibit the arrest of protein synthesis demonstrated with the intact particle (3, 4). Alkylation of SRP68/72 produces a particle defective in promoting translocation but able to recognize signal sequences and halt elongation (5). Particles in which SRP54 is modified lack all three activities (5); since this protein can be cross-linked to signal sequences (6, 7), these results imply that signal recognition is a prerequisite for translation arrest and translocation promotion. In order to investigate the role of SRP RNA, and to approach the function of signal recognition particle *in vivo*, we and others initiated studies in the genetically tractable fission yeast *Schizosaccharomyces pombe* (8, 9, 10).

After showing by one-step disruption of the *SRP7* gene that fission yeast SRP RNA is essential for cell viability (8, 10), we used site-directed mutagenesis to demonstrate the biological importance of a structural motif which lies at the center of the most highly conserved domain in the RNA (11). An additional consideration in the selection of this mutagenesis target was that it encompassed a putative protein binding site. 'Footprinting' experiments had demonstrated that residues in and near each tetranucleotide loop capping the two hairpins in both mammalian and fission yeast SRP RNA are protected from RNAse digestion by the canine SRP19 protein (9, 12). Several non-contiguous regions in both RNAs are protected from RNAse digestion by

the canine SRP68/72 heterodimeric protein (9, 12). The canine SRP9/14 heterodimer associates with the so-called *Alu* terminal domain of mammalian SRP RNA (4, 13, 14) and protects a conserved segment near the 5' end from hydroxyl radical cleavage (15). These footprinting studies suggested a model of SRP organization in which all of the proteins contact the RNA with the exception of SRP54, which would only associate with 7SL in the presence of SRP19 (12). However, more recent experiments conducted under different ionic conditions indicate that mammalian SRP54 protein can bind higher eukaryotic SRP RNAs on its own (16, 17). Mammalian SRP54 also binds to *E. coli* 4.5S (18, 19), which is homologous to Domain IV of SRP RNA (20, 21; nomenclature according to 21); thus, it is likely that this helix is the binding site for SRP54 within the mammalian particle as well.

A class of residues likely to be functionally important in SRP RNA, in addition to those which serve as protein recognition elements, are nucleotides involved in critical RNA-RNA interactions. A phylogenetically conserved secondary structure model has been derived by comparison of homologs from diverse organisms to reveal compensatory base changes (most recently reviewed in 22). All SRP RNAs contain a long central helix in which the 3'-terminal portion is paired with nucleotides near the 5' end. The central portion of the RNA folds into two stem-loop structures, except in eubacterial homologs, which are smaller and form a single hairpin. One or more stem-loops, which in some cases may form a tRNA-like structure, are found at the 5' end of SRP RNAs, with the exception of *E. coli* 4.5S. Finally, some potential long-distance interactions have been suggested on the basis of phylogenetic comparison (22, 23). In addition to stable intramolecular hydrogen bonding, it has been proposed that SRP RNA may undergo conformational changes during the particle's functional cycle (23, 24).

A final category of potentially important nucleotides in SRP RNA are residues which may interact with other components of the SRP cycle. Notably missing from the list of functions mapped onto the particle is ribosome binding. A specific model has been advanced for the interaction of 7SL with rRNA (25), and there is both genetic and biochemical evidence to indicate that the function of *E. coli* 4.5S RNA is intimately linked to protein synthesis (26 — 28). Another possibility is that SRP RNA might bind a region of the SRP receptor which is rich in charged amino acids and resembles nucleic acid binding proteins (29).

As a first step in constructing a functional map of fission yeast SRP RNA, we extensively mutagenized the *SRP7* gene. Our results indicate that this RNA is, on the whole, remarkably insensitive to nucleotide substitutions. Interestingly, most lesions which result in lethal or conditional phenotypes map to the presumptive binding sites of SRP proteins.

MATERIALS AND METHODS

Materials

Restriction enzymes, Klenow fragment, and T4 DNA ligase were purchased from BRL. Reverse transcriptase was from Life Sciences, Inc. T7 DNA polymerase (Sequenase, version 2.0) and other DNA sequencing reagents were from United States Biochemicals. Reagents used for a subset of site-directed mutagenesis reactions were part of a kit from Amersham. Radioactively labeled compounds ($[\gamma\text{-}^{32}P]$ ATP and $[\alpha\text{-}^{32}P]$ dCTP) were from ICN Pharmaceuticals Inc. Chemicals for supplementing liquid media and plates were from Sigma.

Glusulase was purchased from Sigma or DuPont and Novozyme from NovoIndustry. Glass milk, NaI solution, and NEW wash were from Bio 101. Sequencing primers and mutagenic oligonucleotides were synthesized at the University of Illinois Biotechnology Center.

Plasmids

The phagemid pWEC10, which contains the *SRP7* gene on a *Pvu*II fragment that also includes pUC18 DNA sequences at each end, was previously described (11). To circumvent the problem of deletions during mutagenesis of this phagemid (presumably arising via recombination between direct repeats), we constructed two additional phagemids. First, a fragment carrying the *S. pombe SRP7* gene bounded by *Eco*RI and *Sty*I sites was isolated from pWEC10, rendered blunt-ended by filling in with Klenow fragment, and inserted into the *Hinc*II site of pTZ18U to construct pWEC3.2. This fragment was then excised with *Pst*I and *Xba*I and inserted into the same sites in pIRT2 (30) to produce pWEC4.2.

Random mutagenesis

Single-stranded pWEC4.2 DNA template was prepared from *E. coli* strain BW313 using helper phage M13KO7 (31); the strand complementary to the RNA sequence is packaged. Random mutagenesis was carried out using a simple, yet highly efficient technique we developed which employs a high ratio of one nucleotide to the other three to enhance the error rate of either of two DNA polymerases which lack proofreading activity (32). Reactions were performed as previously described except that in some cases both T7 DNA polymerase and AMV reverse transcriptase were employed to achieve a more diverse array of nucleotide substitutions. The mutagenesis primer was 7STx (5' TCGCACTGCCCAAGAC 3'), complementary to nucleotides 183 to 198 of the *SRP7* gene. Random mutations were identified by DNA sequencing prior to phenotypic analysis.

Site-directed mutagenesis

As a prerequisite to future cassette mutagenesis on the *SRP7* gene, four unique restriction enzyme sites were created inside the coding sequence. Single-stranded pWEC10 template used in these reactions was prepared from *E. coli* strain NM522. Five mutagenic oligonucleotides were employed: *Xho*I (5' GACGT-GGCAGCTCGAGGGATC 3'), to create the mutation U88G; *Dra*III (5' CTACACGGTGTGACTGCCAGT 3'), to create U133C,A135C; *Apa*I (5' CGCTATCGCAGGGCCCAA-GAC 3'), to create A192C,G193C; and *Nco*I (5' TACTT-CCATGGACATCCTGC 3'), to create G226C. The presence of mutations was confirmed by digestion with the appropriate restriction enzyme. A four base pair insertion was subsequently created at the *Xho*I site by filling in with Klenow fragment and religating. Additional sequences were inserted by opening up at *Xho*I, blunting with mung bean nuclease, and ligating in the presence or absence of *Pst*I linkers.

For site-directed mutagenesis to identify potential temperature-sensitive mutations (see Results), we employed the 7S-Box B oligonucleotide (5' GAGGGATC*GAAAGCG-A*G*ACTCG-AAC 3'), complementary to nucleotides 61 to 85; starred positions were 37.5% degenerate. For site-directed mutagenesis to pinpoint an osmotic/temperature-sensitive mutation near the 5' end of the RNA, we employed the oligonucleotides A4,A9 (5' CGACCAAGC[C/T]ATTA[C/G/T]AGCGAATAC 3') and A4 (5' CGACCAAGC[C/T]ATTATAGCGAATAC 3'),

Table I. Analysis of randomly distributed point mutations in *S. pombe* SRP RNA. With a few exceptions, mutants are grouped according to their phenotypes. Within each category, alleles are arranged according to the number and type of mutations they contain and the order of occurrence (5′ to 3′) of the first mutation.

Allele[1]	Viability[2]	OTS[3]
G72A,G78A,G100A,G107A,G112A,G141A	Temperature-sensitive	
G153A,G155A	Lethal	
G160A,C164A	Lethal	
G155A,G156A,G160A	Lethal	
A146U,G159U,D148−150	Lethal	
Δ159−161[4]	Lethal	
Δ139−144[4]	Lethal	
Δ118−120[4]	Viable	
G4A,G9A	Viable	+ + +
G4A[5]	Viable	+ + +
G4C[5]	Viable	−
G9A[5]	Viable	+ + +
G100A,C103A,G112A	Viable	−
G156A,G159A	Viable	+ +
U167G	Viable	+
G149A,G152A	Viable	+
A163U,C169U	Viable	+
C180U,Ω176U[6]	Viable	+
G139C,G144C,A162C	Viable	+
U24C,U45C,U62C,U119C,G144C	Viable	+
G15A	Viable	+
C69U[7]	Viable	−
U70C[7]	Viable	−
C77A	Viable	−
G78A[7]	Viable	−
U88G[8]	Viable	−
U101C	Viable	−
G104A	Viable	−
G118A	Viable	−
G121C	Viable	−
U142C	Viable	−
G144C	Viable	−
G149A	Viable	−
Δ149[4]	Viable	−
A170C	Viable	−
A172U	Viable	−
U173C	Viable	−
C174G	Viable	−
A175G	Viable	−
C180U	Viable	−
C180G	Viable	−
C180A	Viable	−
G181C	Viable	−
G182A	Viable	−
G226C[8]	Viable	−
G60A,G61A	Viable	−
C69U,U70C[7]	Viable	−
C69A,U70C[7]	Viable	−
C69A,G127A	Viable	−
U101C,U140C	Viable	−
U119C,U126C	Viable	−
U133C,A135C[8]	Viable	−
C138G,A150G	Viable	−
G139A,G141A	Viable	−
G141A,C148A	Viable	−
G144C,G149C	Viable	−
C179U,C180U	Viable	−
C180A,G181A	Viable	−
A192C,G193C[8]	Viable	−
T(−5)C,T(−3)C,U88C	Viable	−
U12A,U13A,U47A	Viable	−
U57C,U62C,U90C	Viable	−
U88G,U133C,A135C[8]	Viable	−
U101C,A136C,G149C	Viable	−
T(−61)C,G(−75)C,U62C,U101C	Viable	−
U88G,Ω84UCGA[9]	Viable	−
U88G,Δ84−88/Ω131 nucleotides[10]	Temperature-sensitive	−
U88G,Δ84/Ω8 nucleotides[11]	Lethal	

complementary to positions −6 to 18; bracketed positions were equal mixtures of the indicated nucleotides. Each mutation was confirmed by DNA sequencing.

Yeast transformation

The recipient was *S. pombe* strain RM2a, which is heterozygous for disruption of the *SRP7* gene (8). Transformation of plasmid-borne alleles was performed as previously described (11).

Random spore complementation tests

This procedure was performed essentially as described previously (11) except that 5 μl of filtered Glusulase was used for each ascal wall digestion, which was carried out in distilled water.

Determination of growth rates

Growth rates were determined by monitoring optical density. Haploid strains were grown in rich liquid medium (YEL) at 30°C. Where appropriate, a mutant and control isogenic wild-type culture were simultaneously shifted to 37°C to test for temperature sensitivity.

Detection of osmotically induced temperature sensitivity

Ura$^+$ Leu$^+$ haploid h$^+$ colonies carrying mutant *SRP7* genes on an extrachromosomal plasmid and the disrupted gene in the chromosome were identified after random spore analysis and tested for sensitivity to 20% or 25% glycerol at 37°C as previously described (11).

RESULTS

Distribution of mutations in SRP RNA

We analyzed fifty-two mutant alleles obtained by random point mutagenesis, seven alleles derived from site-directed mutagenesis to introduce restriction sites into the *SRP7* gene, nine alleles designed to pinpoint conditional lesions (see below), and three alleles with extra nucleotides inserted at position 84; these are listed in Table I. In the random pool, there were 21 single, 15 double, and 10 multiple mutants, as well as 5 deletion mutants and a single insertion. The nucleotides mutated extend from −75 in the 5′ flanking DNA to position 182 in the RNA coding sequence; no mutations were isolated in the 71 nucleotides at the 3′ end of the *SRP7* gene because the random mutagenesis

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[1] Generated by random mutagenesis (see Materials and Methods) unless otherwise noted.
[2] Assessed by random spore analysis (see Materials and Methods).
[3] Osmotically induced temperature sensitivity: +, grows on YEA plates containing 25% glycerol at 37°C, but more slowly than wild-type; + +, a serious growth defect, more pronounced on plates containing 25% than 20% glycerol; + + +, unable to grow at high temperature even on 20% glycerol; −, no apparent osmotic inhibition of growth relative to a wild-type strain.
[4] Δ = Deletion of the specified nucleotide(s).
[5] Generated by site-directed mutagenesis to pinpoint an OTS mutation (see Materials and Methods).
[6] Ω = Insertion of the indicated nucleotide(s) 5′ to the position specified.
[7] Generated by site-directed mutagenesis to assay potential TS mutations (see Materials and Methods).
[8] Generated by site-directed mutagenesis to create restriction enzyme sites within the SRP RNA coding sequence (see Materials and Methods).
[9] Generated by religating the filled-in *Xho*I site (see Materials and Methods).
[10] Derived from a cloning accident through which a portion of the *SRP7* gene (positions 164−236) plus 59 nucleotides of vector sequence were inserted at the *Xho*I site which was blunted with mung bean nuclease.
[11] Generated by insertion of the sequence 5′ GCTGCAGC 3′ (a *Pst*I linker) at the *Xho*I site from which one nucleotide was removed with mung bean nuclease.
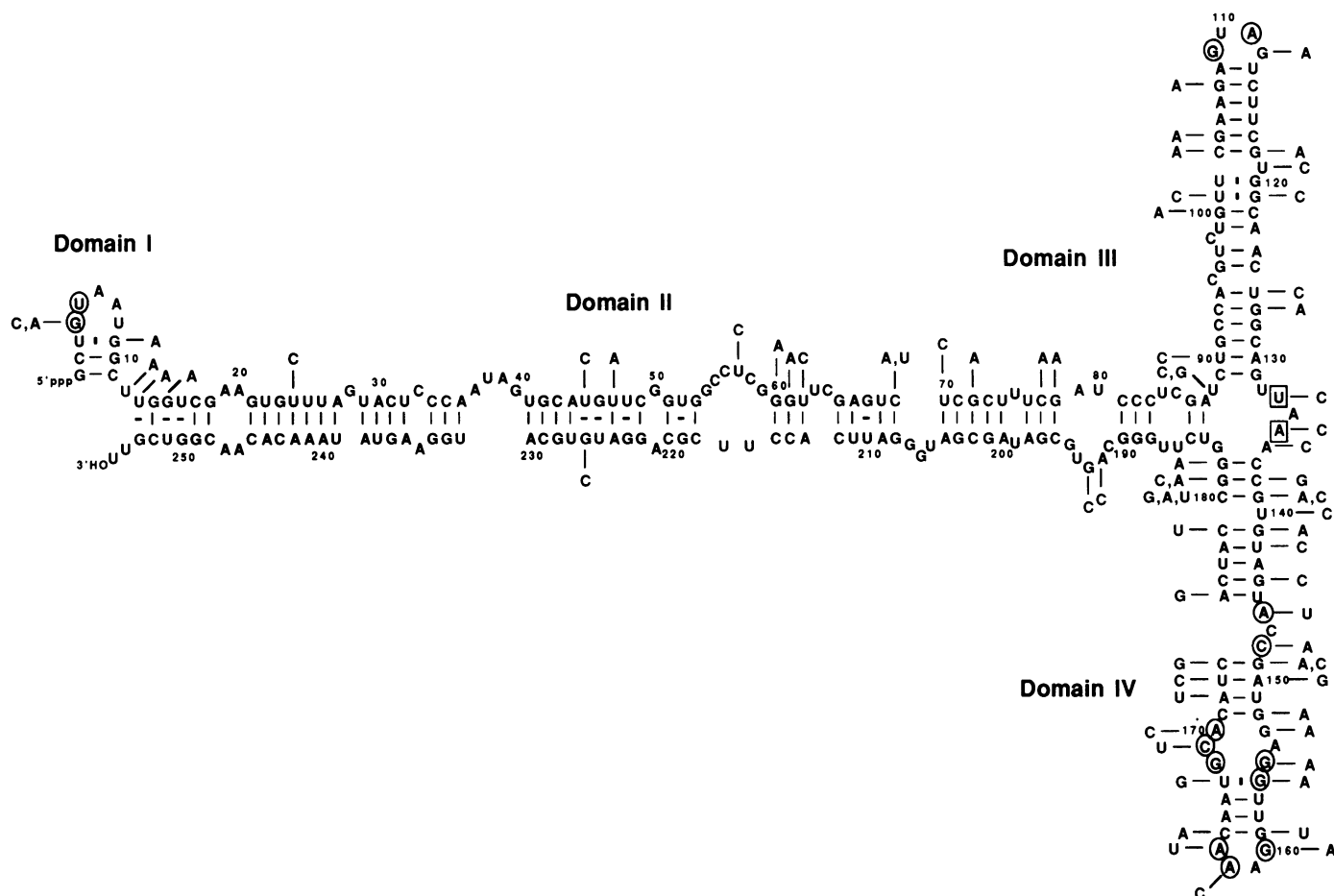
Figure 1. Locations of randomly distributed point mutations within the fission yeast SRP RNA secondary structure. A composite secondary structure is shown in which aspects of the models proposed by Brennwald *et al.* (8) and Poritz *et al.* (9) are incorporated. Residues which are >90% conserved among all SRP RNA homologs where the domain is present (22) are circled. Residues which are >90% conserved among eukaryotic SRP RNAs are boxed. Only point mutations are indicated in the figure.

primer ends at position 183. Although the positions nearest the primer are most heavily mutated, the substitutions are spread over approximately 200 nucleotides, as we previously reported for this technique (32). The locations of point mutations from all three pools are indicated on the 7SL secondary structure shown in Figure 1. Of the 255 nucleotides in the RNA, 66 were substituted in one or more of these alleles.

Alteration of highly conserved nucleotides often produces no phenotypic effects

Phenotypic analysis of mutant SRP RNAs was performed using the complementation assay we previously described (11); the results are shown in Table I. Mutant alleles were scored first for viability using random spore analysis, then for cold and heat sensitivity (by incubation at 18°C and 37°C, respectively), and finally for growth impairment at high temperature on high osmotic strength medium (see Materials and Methods and Footnote 3 under Table I).

Most of the 7SL mutants we analyzed showed no apparent phenotypic effects relative to wild-type cells under any condition tested. Although SRP RNA as a whole shows limited primary sequence conservation through evolution, Domain IV contains several invariant residues. No growth defects were observed

under any growth condition tested for three alleles in which nucleotides conserved among a variety of eukaryotic, archaebacterial and eubacterial RNAs homologous to SRP RNA were altered. These mutants are [G141A,C148A], [A170C] and [A175G]. The [U133C,A135C] allele, which carries substitutions in two residues conserved among all eleven sequenced eukaryotic SRP RNAs, was also phenotypically silent. Two other alleles with alterations in universally conserved residues, [G153A,G155A] and [A163U,C169U], produced only mild conditional impairment of growth.

The putative transcriptional regulatory regions designated Boxes A and B (9) comprise another class of nucleotides conserved in primary sequence, although these elements are not constant with regard to their positions in the SRP RNA secondary structure. From the random mutagenesis pool, we obtained three mutations which lie within Box B ([G61C], [U62C], and [U69A]), all as part of multiple mutants. None of these had any phenotypic effects despite altering generally invariant residues of the consensus (reviewed in 33). For reasons described below, we wanted to make additional mutations near Box B; the mutagenic oligonucleotide was designed to also incorporate changes in the transcriptional control element (see Materials and Methods). One of these was [C69U], which alters the last, highly

conserved, residue of Box B; its growth is wild-type under all conditions tested. Also mutated was U70, which is conserved between *S. pombe* and *Y. lipolytica* SRP RNAs (9); since the consensus at this position is a C (33), it is not surprising that [U70C] produced no phenotypic effects. The double mutations [C69U,U70C] and [C69A,U70C] also permitted wild-type growth. Our results are consistent with the observations of previous investigators: although mutations in conserved Box B residues often dramatically reduce RNA polymerase III transcription *in vitro*, they generally have little or no impact on cell growth, with the exception of changes in the central C residue (which were not among our mutants).

Lethal mutations alter conserved sequences and structures

Only three of the sixty-one alleles carrying point mutations exhibited lethal phenotypes. Although two of these contain a G→A substitution at the invariant first residue (nucleotide 160) of the Domain IV tetraloop, our previous work showed that this mutation is not lethal on its own (11). In [G160A,C164A], the last base pair of the phylogenetically proven stem is also disrupted. The additional mutations in [G155A,G156A,G160A] alter two conserved residues within Domain IV. Both of these are single-stranded in the commonly accepted secondary structure (reviewed in 22), but G156 can form a G·U pair with position 167 in the *S. pombe* RNA (see Fig. 1), which would become an A-U in the mutant; a potential A-U pair is found in the same location in *Y. lipolytica* SRP RNA, and a G*A pair can be formed in all other homologs. The third lethal mutant is [G153A,G155A], which contains transitions in two nucleotides within the terminal bulge of of Domain IV; it seems likely that the lethality is a consequence of changing G155, since this residue is highly conserved through evolution and is in common with one of the bases changed in the lethal triple substitution mutant.

The three other lethal alleles isolated from the random mutagenesis pool contain small deletions in Domain IV. One, [A146U,G159U,Δ148−50] contains two point mutations in addition to the three-nucleotide deletion; we have shown in independent work that [G159U] produces a severe conditional phenotype on its own (34). Since point mutations in and near the Domain IV tetraloop are often lethal (11), it is not surprising that the deletion of nucleotides 159−161 produced an RNA that cannot support viability. The lethality of a five-base deletion near the top of the Domain IV helix is likely due to perturbation of the secondary structure of this region of the RNA. A three-nucleotide deletion in the middle of Domain III produced a severe conditional phenotype, but the cells were viable, suggesting that there may be fewer constraints on this hairpin than on Domain IV.

Some insertions are tolerated at the junction of Domains II and III

Another location where gross changes have been introduced is position 84, which lies at the junction of two of the three helices which comprise the core of the SRP RNA secondary structure. An insertion of four nucleotides produced by filling in an *Xho*I site yields no detectable phenotype, while insertion of an eight nucleotide *Pst*I linker combined with the deletion of nucleotide 84 (see Footnote 10, Table I) was lethal. Surprisingly, a much larger insertion at this position was tolerated under normal growth conditions; the 131 nucleotides introduced included duplication of a portion of the RNA coding sequence (see Footnote 9, Table I). Cells which carry this allele as their only source of SPR RNA were, however, inviable at high temperature. The cessation of

growth in this mutant did not occur until ca. two generations after shift to 37°C (data not shown), similar to what we observed for a temperature-sensitive point mutant described below.

Many perturbations of base pairing are tolerated

Since SRP RNA is highly structured, the majority of the lesions we introduced are located in base-paired regions. The single mutants [C77A], [U142C], [A172U], [U173C], [G181C] and [G226C], which carry substitutions in the middle of short base-paired regions, had no phenotypic effects under any condition tested. In addition, many multiple mutants which include several such changes also produced no growth defects. Thus, the resulting non-Watson-Crick appositions may not significantly destabilize the helices, perhaps due to individual secondary structure elements being coaxially stacked or otherwise constrained by the overall conformation of the RNA; it is also conceivable that bound proteins stabilize the RNA structure.

Osmotically induced temperature sensitivity arises from mutations distributed throughout SRP RNA

We previously described an unusual, but not unprecedented, conditional phenotype arising from mutations in fission yeast SRP RNA: the inability to grow at high temperature on high osmotic strength medium (11), which we designated the OTS phenotype. All of the mutations examined in our earlier work were localized to the Domain IV tetraloop region. Interestingly, the random mutant pool includes a number of mutations located elsewhere in the RNA which confer a similar conditional growth defect. One of the three severe OTS mutants, described above, has a small deletion in the Domain III stem. A triple point mutant which affects residues in Domain III is moderately OTS.

A second severe OTS mutant, [G4A,G9A], contains two transitions located very near the 5′ end of SRP RNA. Both substitutions lie within the region of similarity to the RNA polymerase III transcription element designated Box A (9). In addition, the first mutation alters the third of seven conserved residues which, in the human RNA, lie within the region protected from hydroxyl radical cleavage by the SRP9/14 heterodimer (15). To test whether this change alone was responsible for the observed phenotype, we created alleles of *SRP7* which carried [G4A], [G9A], and [G4C]; the last change was designed to prevent hydrogen bonding between positions 4 and 8 (see Fig. 1). Unexpectedly, a conditional phenotype was not observed for either [G4A] or [G9A], while [G4C] was OTS. The fact that growth impairment was observed upon changing position 4, which is flexible in the RNA polymerase III Box A consensus, but not position 9, which is a G (33), argues against a defect in synthesis of the RNA being responsible. The effect of the double mutant is very likely a consequence of an aberrant secondary structure (see Discussion).

With one exception, the seven mild OTS mutants contain exclusively alterations of residues in Domain IV. In two cases, [G156A,G159A] and [A163U,C169U], at least one of the mutations falls in a highly conserved residue. The [U167G] mutant allele is altered in a position which is invariant in SRP RNAs with the exception of the fission yeast homolog; the base change does not restore the consensus. [G149A,G152A] disrupts the first and last Watson-Crick pairs in a helical segment near the center of Domain IV, while [C180U,Ω176U] disturbs the continuity of pairing toward the junction of this domain with Domains II and III. The phenotype of [G139C,G144C,A162C] is the same as that of [A162C] alone; thus, its conditional growth
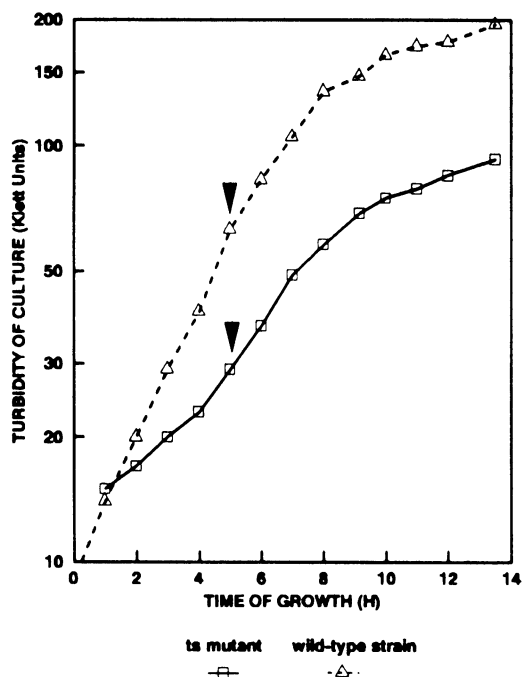
Figure 2. Growth curves of the temperature-sensitive sextuple point mutant and an isogenic wild-type strain. After five hours of growth at 30°C, both cultures were shifted to 37°C and incubated for an additional 9 hours. The filled arrow indicates the time of the temperature shift.

defect is probably caused by changing this residue, which is invariant among SRP RNAs except those of plants. The last mild OTS allele, [U24C,U45C,U62C,U119C,G144C] is perplexing, since the changes are distributed over Domains II, III, and IV and none of the altered residues are conserved, nor is any individual mutation predicted to disrupt secondary structure significantly.

A temperature sensitive allele with six point mutations is most likely assembly-defective

One of the reasons for adopting a random mutagenesis approach was that we previously found no *bona fide* temperature-sensitive (TS) alleles among sixteen site-directed SRP RNA mutants (11). The random pool did contain one allele which confers a growth defect at high temperature even at normal osmolarity; it carries six point mutations (see Table I). The paucity of TS mutants may be, at least in part, due to the highly structured nature of this RNA, i.e. substitutions of individual nucleotides (as opposed to deletions or insertions) may not significantly perturb its thermal stability or assembly with proteins.

In an initial effort to distinguish whether the temperature sensitivity of the TS strain is due to a defect in the function of SRP RNA, as opposed to its synthesis or assembly into a particle, we performed the growth experiment shown in Figure 2. After a shift from 30°C to 37°C, the mutant continued to double at the 30°C rate for ca. 2.5 hr, approximately one generation time, and then growth slowed, but did not cease. None of the six point mutations in this gene are located in the putative RNA polymerase III transcription signals (9); moreover, although the half life of fission yeast SRP RNA has not been directly measured, its mammalian counterpart is known to be stable. These facts argue against the leveling off of growth after only a single generation

at the non-permissive temperature being due to a defect in synthesis of new SRP RNA. As is also evident in Fig. 2, the TS strain grows slightly slower than an isogenic wild-type strain at 30°C, suggesting that the mutant RNA may not be fully functional even at the permissive temperature. Taken together, the growth properties conferred by the mutant RNA suggest that its assembly with one or more SRP proteins may be impaired, perhaps due to aberrant folding or alteration of one or more critical residues. The observed decrease in growth rate approximately one generation after the shift to the non-permissive temperature may reflect a combination of further destabilization of already defective particles containing RNA made at 30°C, and their dilution with SRP containing the more significantly defective RNA synthesized at 37°C. As a second approach to gaining insight into the TS defect, we tested whether growth would resume upon shifting the cells from the non-permissive to the permissive temperature. After a 24 hour incubation at 37°C, 500 to 800 mutant cells were spread onto two YEA plates which were then incubated separately at 30°C and 37°C for two days. No colonies were observed on the plate incubated at high temperature and only six colonies grew on the plate incubated at low temperature. These results suggest that cell death occurs at a very high frequency under non-permissive conditions. The apparent irreversibility of the TS defect is consistent with the mutant RNA producing an unstable SRP even at a temperature that permits growth.

It was also of interest to locate the nucleotide substitution(s) which conferred the TS defect on this strain. To determine whether any of the mutations in this allele could alone suffice to produce the TS phenotype, we first examined the remainder of the random mutagenesis pool for overlaps. [G141A] was isolated twice independently as one of two mutations in alleles which resulted in no phenotypic effects under any conditions examined. [G100A] and [G112A] are two of the three mutations in an allele which is moderately TS at high osmotic strength, but not on standard media. We next generated [G78A] by site-directed mutagenesis with the Box B oligonucleotide (see Materials and Methods) and found that it was phenotypically silent. Of the two remaining mutations in the TS allele, [G72A] seems unlikely to be solely responsible for the phenotype, since it lies within an extended and very stable helix (8). Unfortunately, despite extensive efforts with several different oligonucleotides, we were unable to create site-directed mutations at position 107 or at other positions in this region of the RNA (35). We believe that this is due to the fact that this nucleotide lies within a 6 base pair helix capped by a GAAA loop, one of two sequences known to stabilize RNA hairpins (36); their presence in even short oligonucleotides can increase the T_m by ca. 20°C (37, 38). Presumably either the single-stranded DNA template, the mutagenic oligonucleotides, or both, form stable intramolecular structures and fail to interact. Since [G107A] disrupts the penultimate base pair (a G-C) of this RNA helix, thereby dramatically reducing its stability, it is a likely candidate for conferring the growth defect at high temperature; it may, however, act in concert with [G112A] (see Discussion).

DISCUSSION

A highly efficient method we developed for random mutagenesis (32) allowed the rapid isolation of a large number of mutant alleles of the *SRP7* gene. Phenotypic analysis revealed that, remarkably, SRP RNA can tolerate nearly any mutation introduced; the
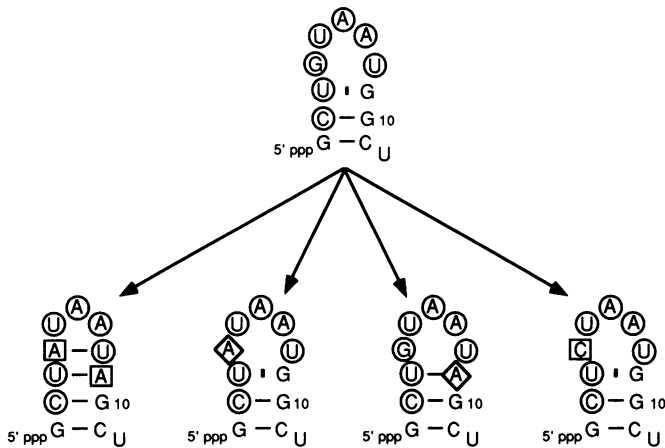
Figure 3. Effects of mutations in the SRP9/14 protein binding site. At the top is shown the 5' end of fission yeast SRP RNA, with conserved residues corresponding to those protected from hydroxyl radical cleavage in the human RNA (15) circled. The sequence and predicted secondary structure changes in the four mutant alleles we analyzed in this region are shown on the second line. Bases substituted in mutant alleles which confer conditional growth defects are surrounded by a square, while those which allow wild-type growth are surrounded by a diamond.

exceptions generally lie in conserved residues which, in the mammalian RNA, are protected from nucleolytic agents by SRP proteins. In the following discussion, the mutants we analyzed are grouped according to their functional implications.

Mutations which affect the SRP9/14 protein binding site

It was recently shown that, in mammalian SRP RNA, nucleotides near the 5' end are protected from ribonuclease digestion by the canine SRP9/14 heterodimeric protein (15). The high degree of sequence conservation in a seven nucleotide stretch within this region (22) suggests that it might serve as a binding site for homologs of these proteins in other organisms. Comparison of all available SRP RNA sequences indicates that the most highly conserved nucleotides protected by the protein are single-stranded (15). In higher eukaryotic homologs, these are bulged between two helical segments in a proposed tRNA-like structure, while in lower eukaryotic SRP RNAs, they form the loop of a single hairpin. The phenotypes of four *SRP7* alleles we analyzed which carry mutations near the 5' end of the fission yeast RNA are consistent with the idea that these changes lie within the SRP9/14 binding site. Their effects substantiate the notion that the most highly conserved residues must be single-stranded, and point to the importance of one nucleotide in particular as a potential point of protein contact. The positions of sequence changes and their predicted effects on secondary structure are summarized in Figure 3. Unexpectedly, while cells carrying the [G4A,G9A] allele as their only source of SRP RNA were unable to grow at 37°C on high osmotic strength medium, neither [G4A] nor [G9A] alone produced a discernible conditional growth defect. Since G9 lies outside the conserved region, the lack of effect of a transition here is not surprising. G4 is, in contrast, highly conserved from archaebacteria to humans; however, an A is sometimes found at this position, albeit rarely (3/32 cases; 22). The changes in the double mutant are predicted to increase the stability of the 5' stem by replacing a G·U pair with an A-U and adding another A-U pair (see Fig. 3); this would place the

A residue at position 4 in a base-paired, rather than single-stranded, configuration. We therefore suggest that the conditional lethality of this mutant is primarily due to secondary structure perturbation. A transversion at position 4 resulted in a severe conditional growth defect, supporting the idea that this residue may be a sequence-specific contact for a fission yeast homolog of the SRP9/14 heterodimer. Thus, our data suggest that both the identity of the residue and the structure at position 4 are critical for protein binding. Specifically, what is required for wild-type SRP RNA function appears to be a single-stranded purine.

Mutations which affect the SRP19 protein binding sites

The SRP19 protein protects from RNAse digestion residues in and near the tetranucleotide loops capping both the Domain III and Domain IV helices in mammalian SRP RNA (12). Similar regions of fission yeast SRP RNA are also protected by the canine protein (9). In addition to the present work, we have virtually saturated the Domain IV tetraloop with single substitutions and made several *en bloc* replacements by site-directed mutagenesis (11; 34). The results of these experiments demonstrate that sequence changes are tolerated in this region of fission yeast SRP RNA as long as a proper structure can be formed; the requirements for a functional RNA are a stabilizing tetranucleotide loop flanked by any base pair. The lethal mutations in the Domain IV tetraloop described here reinforce this view, since all are predicted to affect hydrogen bonding (including intra-loop interactions; 36) or loop size.

Recent *in vitro* analysis of deletion mutants of human SRP RNA suggests that the primary determinant of SRP19 binding is Domain III, although the presence of Domain IV seems to improve efficiency (39). Among the alleles isolated from our pool of randomly generated fission yeast SRP RNA mutants is the triple base substitution [G100A,C103A,G112A], which exhibits moderate sensitivity to the combination of high temperature and osmotic strength (see Table I). Since the G→A change at nucleotide 112 is within the region protected from RNAse digestion by the canine SRP19 protein (9), disruption of binding to a fission yeast homolog of this protein may be the cause of the observed phenotype. The [G112A] substitution is also found in the sole point mutant that is temperature-sensitive even at normal osmotic strength. In addition to [G112A], it has five base changes distributed across Domains II, III and IV, including the [G100A] substitution which is also in common with the OTS mutant. We have narrowed down the cause of the TS phenotype to the three Domain III mutations (see Results). The relevant difference between this mutant and the one which required elevated osmotic strength as well as high temperature to manifest its growth defect is thus a G→C substitution at position 107. This mutation disrupts the second of six base pairs in the top helical segment of the Domain III stem, significantly destabilizing it. This in turn may alter the conformation of the tetranucleotide loop, the most likely site of SRP19 protein binding.

Mutations which may affect SRP54 protein binding

As noted in the Introduction, the canine SRP54 protein binds *E. coli* 4.5S RNA, which is homologous to Domain IV of mammalian SRP RNA (16, 17), suggesting that this region serves as the protein binding site in the mammalian particle as well. However, RNAse protection or other experiments have yet to be carried out to confirm that this is indeed the case. Although no mapping data exist as yet, the conserved residues lying in bulged segments of Domain IV (see Fig. 1) are excellent
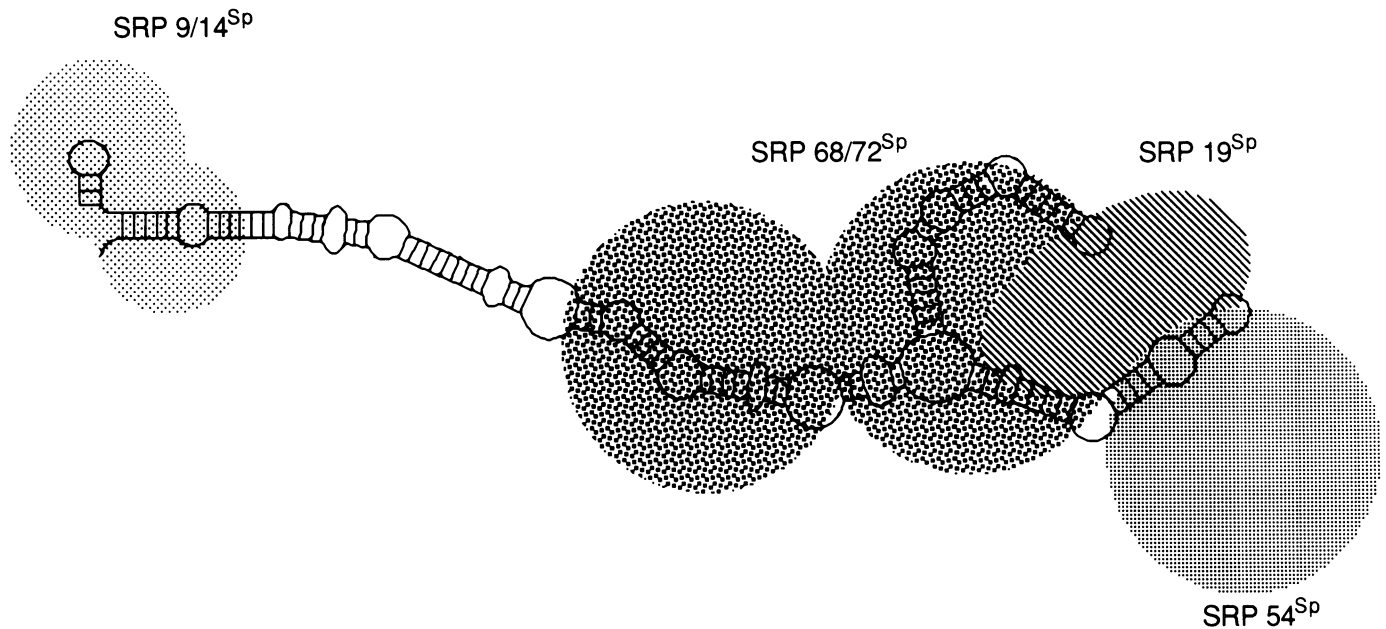
Figure 4. Model of fission yeast SRP organization. The protein subunits of the particle, identified in a different study from our laboratory (40) are shown arranged in their most likely configuration, based on the present analysis of mutations in SRP RNA, earlier work in which canine SRP proteins were footprinted onto the fission yeast RNA (9), and analogy to mammalian SRP (12, 15).

candidates for sequence-specific protein contacts. Several alleles from our random pool of fission yeast SRP RNA mutants which had phenotypic consequences carried mutations in these nucleotides. Most severe were the lethal mutants [G153A,G155A] and [G155A,G156A,G160A]. Our previous work showed that [G160A] alone produces a viable but moderately OTS cell (11). We have recently obtained evidence, to be published elsewhere, that the [G155A,G156A,G160A] triple mutant is unable to bind the SRP54 protein, while [G160A] shows no significant defect (34). Taken together, these results suggest that both of the conserved G residues in the 3' terminal bulge of Domain IV (see Fig. 1) are likely to be involved in fission yeast SRP54 protein binding. The mild conditional phenotype of the [U167G] mutant may also arise from perturbing interactions with this protein binding, since this residue is directly across the helix from (and potentially pairs with) G156 (see Fig. 1).

A mutation which may affect SRP68/72 protein binding

The canine SRP68/72 heterodimeric protein protects from RNAse digestion a diffuse segment extending over much of Domains II and IV in both the fission yeast and mammalian RNAs; in addition, it protects the central portion of Domain III in the mammalian, but not the *S. pombe*, RNA (9, 12). All of the eleven alleles we isolated carrying nucleotide substitutions exclusively in Domain II are phenotypically silent, despite the fact that three of the mutations occur in residues which are protected by the protein (A60, A192 and G193). The lack of effect upon mutating these nucleotides may indicate that they do not interact directly, or at least not in a sequence-specific manner, with the protein. Alternatively, the protein may make multiple contacts with the RNA, such that single mutations do not dramatically reduce its affinity. Fourteen alleles carrying point mutations in the region of Domain IV protected by the SRP68/72 heterodimer also confer

no discernible phenotypes; however, in this case none affect residues protected by the protein. The only remaining mutant whose phenotype may be due to perturbing SRP68/72 binding is [C180U,Ω176U]; its mild sensitivity to elevated temperature and osmotic strength may be due to inserting a nucleotide across the helix from several residues protected by the protein, thereby perturbing the RNA conformation. [C180U] was isolated independently (see Table I) and is phenotypically silent.

RNA-RNA interactions

Several years ago, a model was presented for dramatic structural reorganization occurring at the junction of the three major helices in mammalian SRP RNA during the SRP cycle (24). Because this model was based on the observation that linker insertion into the core region produced structural perturbations, manifested as altered mobilities on native gels, these results can be interpreted alternatively as implying that the inserted linker can form base pairs with another part of the RNA, thereby changing its overall structure. Since we have analyzed a large number of mutations in the corresponding region of fission yeast SRP RNA and found virtually all of them to be without phenotypic consequences, our data cast doubt on the structural reorganization model. In addition, the region proposed to be involved in this 'switch' has since been shown to be protected from RNAse digestion by the SRP68/72 protein (12), also arguing against such an extensive helical rearrangement. More recently, chemical probes were used to assess the accessibility of individual nucleotides in naked SRP RNA, free signal recognition particle, polysome-bound SRP, and membrane-bound SRP (23). Increases in sensitivity to chemical modification during progression through the SRP cycle were taken to indicate conformational changes in 7SL RNA. It is difficult to assess the *in vivo* relevance of these observations, since the isolation procedures for each form of SRP required buffers

of different ionic strength, which could influence both RNA conformation and, especially, RNA-protein interactions. Indeed, the nucleotides whose modification patterns were altered were not localized, but distributed throughout the molecule in each case. Our data do not rule out conformational changes in SRP RNA. However, the lack of mutations whose phenotypes cannot be explained as resulting from disruption of RNA-protein interactions suggests that small shifts in base pairing, as predicted to result from many of the silent mutations, have undetectable effects on the activity of the particle.

Conclusion

While we have by no means saturated the fission yeast *SRP7* gene with mutations, the extensive roster described here allows at least a preliminary functional map of SRP RNA to be constructed. As noted throughout the text, direct biochemical analysis of RNA-protein interactions between fission yeast SRP RNA and homologous proteins has not yet been undertaken. However, native immunoprecipitation experiments with antisera directed against *S. pombe* SRP54 (to be published elsewhere) indicate that the protein composition of fission yeast SRP is similar to that of its mammalian counterpart; that is, six polypeptides are present whose sizes differ only slightly from those of the individual subunits of mammalian SRP (40). In addition, published work from another laboratory indicates that both the canine SRP19 and SRP68/72 proteins protect from ribonuclease digestion similar regions in mammalian and fission yeast SRP RNAs (9). Assuming that binding sites for the remaining proteins are also homologous, the mutagenesis data presented here suggest that the role of SRP RNA which is most susceptible to mutational perturbation is that of a scaffold for ensuring the proper three-dimensional arrangement of SRP proteins. Taken as a whole, these results suggest the working model of fission yeast SRP organization presented in Figure 4. Detailed analysis of the effects of mutations in putative protein binding sites, as well as other perturbations of SRP RNA structure, should allow our picture of RNA-protein interactions in SRP to be refined, as well as perhaps identifying critical intra- and intermolecular RNA-RNA interactions.

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