UNIVERSITY OF ILLINOIS

May 7 19 93

THIS IS TO CERTIFY THAT THE THESIS PREPARED UNDER MY SUPERVISION BY

Wendy R. Davis

ENTITLED Mutational Analysis of a Proposed Structural Element in the

SRP9/14p Binding Site of Schizosaccharomyces pombe SRP RNA

IS APPROVED BY ME AS FULFILLING THIS PART OF THE REQUIREMENTS FOR THE

DEGREE OF Bachelor of Science in Biochemistry

Instructor in Charge

APPROVED:

HEAD OF DEPARTMENT OF Biochemistry

MUTATIONAL ANALYSIS OF A PROPOSED STRUCTURAL ELEMENT IN THE SRP9/14P BINDING SITE OF *SCHIZOSACCHAROMYCES POMBE* SRP RNA

BY

Wendy R. Davis

THESIS
FOR THE
DEGREE OF BACHELOR OF SCIENCE
IN
BIOCHEMISTRY

COLLEGE OF LIBERAL ARTS AND SCIENCES
UNIVERSITY OF ILLINOIS
URBANA, ILLINOIS

1993

TABLE OF CONTENTS

ACKNOWLEDGMENTS

I would like to first thank Dr. Jo Ann Wise for her generosity in giving me a position in her laboratory for the year, showing me what research in the molecular biology field is like, and showing me the road to a Ph.D. program. I would also like to thank Dave Selinger and Steve Althoff who advised me not only on strategies for my project but in "those little things" that never seem to make their way into a written protocol; and thanks to Chuck Romfo, Consuelo Alvarez, Roger VanHoy, and Claudia Reich who listened to me during my trials and tribulations when I was applying to graduate school. I would like to thank everyone in the Wise lab; they have only strengthened my desire to do research.

INTRODUCTION

Signal recognition particle (SRP) is a cytoplasmic ribonucleoprotein that is necessary for correct protein targeting to the endoplasmic reticulum (ER) *in vitro* (Walter and Lingappa, 1986). Mammalian SRP is an 11S particle composed of six polypeptide chains (Walter and Blobel, 1980) and a 300 nucleotide SRP RNA (Walter and F'obel, 1982). The six polypeptides are organized into two monomeric subunits with molecular weights 19 kd and 54 kd (designated SRP19p and SRP54p respectively) and two heterodimers, one consisting of two polypeptides with molecular weights of 9 kd and 14 kd (designated SRP9/14p) and one whose components have molecular weights of 68 kd and 72 kd (designated SRP68/72p) (Siegel and Walter, 1985).

The current model for SRP function is based on experiments employing an *in vitro* system consisting of canine pancreas microsomes (closed vesicles derived from ER membranes) and purified SRP added to wheat germ translation components. The mechanism is as follows (see Figure 1): when the signal sequence at the amino terminus of a nascent polypeptide emerges from a ribosome translating a protein destined for secretion (Step A), SRP binds to the hydrophobic core and slows or halts further chain elongation (Step B; Walter and Blobel, 1981; Wolin and Walter, 1989). SRP, complexed with the ribosome and nascent polypeptide chain, is

then directed to the endoplasmic reticulum membrane where it interacts with a membrane-bound protein known as the "docking" protein (Meyer *et al.*, 1982) or SRP receptor (Steps C and D; Walter and Blobel, 1981). This receptor consists of two polypeptides: SRαa 72 kd peripheral membrane protein and SRβ a 30 kd integral membrane protein that binds the α subunit (Tajima *et al.*, 1986; Ogg *et al.*, 1992). After binding to the receptor, SRP dissociates, and the ribosome/nascent chain complex is then released from the SRP receptor and binds to other membrane bound complexes such as a signal sequence receptor (Step E; Wiedmann *et al.*, 1987) before translocation. Translocation of the polypeptide then occurs concurrent with the resumption of translation. SRP can then cycle for another round of targeting (Step F).

In the mammalian *in vitro* system, roles have been ascribed to each of the proteins in the SRP cycle, except for SRP19p, by specifically disrupting the function of each of the SRP proteins by alkylating with N-ethyl maleimide (NEM) and then reconstituting SRP. For instance, when SRP68/72p is alkylated, the particle becomes unable to promote translocation but can still effect a translation pause (Siegel and Walter, 1988a). When SRP is reconstituted without SRP9/14, translocation still occurs, but the particle cannot arrest protein synthesis (Siegel and Walter, 1985; Siegel and Walter, 1986). Upon modification of SRP54p, protein translocation, translation arrest and signal sequence recognition

activities are all lost (Siegel and Walter, 1988a). Other studies have shown that SRP54p can be cross-linked to signal sequences (Krieg *et al.*, 1986; Kurzchalia *et al.*, 1986), suggesting that it is the component responsible for signal sequence recognition. The loss of elongation arrest and translocation by SRP reconstituted with an inactivated SRP54 protein is most likely a consequence of the requirement for signal sequence recognition for SRP activity (Siegel and Walter, 1988a).

When an RNA was discovered in the fission yeast *Schizosaccharomyces pombe* (see Figure 2) that is similar to mammalian SRP RNA (Brennwald *et al.*, 1988; Poritz *et al.*, 1988; Ribes *et al.*, 1988), *in vivo* genetic studies became possible. SRP components from many organisms have been cloned; however, genetic and molecular biological manipulations are difficult to perform in mammals. For instance, even though it is possible to introduce modified genes into mammalian cells, it is difficult to ensure proper gene regulation unless a homologous integration of the gene of interest is performed. Fission yeast is ideal for studying SRP: like other yeasts, it grows quickly, has a small genome (only five-fold larger than *E. coli*); and can exist in a haploid or diploid state. However, unlike *Saccharomyces cerevisiae*, a budding yeast in which SRP is not essential for viability and which has an SRP RNA lacking resemblance to that of any other organism (Hann and Walter, 1991),

S. pombe requires SRP for growth (Brennwald *et al.*, 1988; Ribes *et al.*, 1990, Althoff *et al.*, manuscript in preparation).

Although the *S. pombe* SRP RNA has little primary sequence homology to the mammalian SRP RNA, they have a common secondary structure (Brennwald *et al.*, 1988). *S. pombe* SRP is quite similar to mammalian SRP in its behavior on aminobutylagarose and DEAE-Sepharose chromatography (Brennwald *et al.*, 1988; Poritz *et al.*, 1988). Through sucrose gradient analysis, it was determined that *S. pombe* SRP is 11S in size (Brennwald *et al.*, 1988) and contains six polypeptides that are each similar in size to a component mammalian SRP (Selinger *et al.*, manuscript in preparation). RNAse protection studies showed that canine SRP19p and SRP68/72p proteins bind to similar regions in human and *S. pombe* SRP RNA (Poritz *et al.*, 1988; Siegel and Walter, 1988b). Sequences and structural elements important for SRP54p binding have been determined by native immunoprecipitation experiments on mutant *S. pombe* SRP RNAs (Selinger *et al.*, 1993). Most importantly for this particular discussion, it has been shown that the canine SRP 9/14p heterodimeric protein is associated with the Alu region, which consists of the 5' and 3' ends of mammalian SRP RNA (Siegel and Walter, 1985), and that it specifically protects a conserved sequence of seven nucleotides at the 5' end from hydroxyl radical cleavage (Strub *et al.*, 1991).

Experiments from our laboratory, specifically random *in vitro* mutagenesis of the *SRP7* gene followed by phenotypic analysis *in vivo* have shown that most point mutations in *S. pombe* SRP RNA result in wild type behavior when the mutant SRP RNAs are the only source of SRP RNA for the cell (Liao *et al.*, 1992). Of the mutants that showed lethal or conditional phenotypes, most were within putative protein binding sites of SRP proteins. These experiments have led to a model for the proposed binding sites of SRP proteins on fission yeast SRP RNA (see Figure 3).

It has been hypothesized that both the structure of the RNA and the identity of the seven conserved nucleotides within and surrounding the 5' end hairpin loop are critical for binding of a fission yeast homolog of the SRP9/14p heterodimer (Liao *et al.*, 1992). One conditional mutant isolated through random mutagenesis [G4A,G9A] (see Figure 4) alters one of the two invariant residues in this conserved sequence, as well as a nucleotide that lies just outside of the putative protein binding site. Thus, the mutant's conditional phenotype was thought to be due to the G to A transition at position 4. However, the fact that each of the single mutants, [G4A] and [G9A] exhibits wild type growth (see Figure 4) suggests otherwise. An alternative explanation is that the phenotype of the [G4A, G9A] double mutant is due to the increased stability of the 5' hairpin loop. The conditional OTS phenotype of a G to C change at position 4

suggests that, specifically, a single-stranded purine is required at this position (Liao *et al.*, 1992).

The goal of my senior thesis research is to further characterize the SRP9/14p binding site as the first step toward isolating genes encoding the *S. pombe* homologs of these proteins. First, I have tested the model discussed above by carrying out phenotypic analysis of another mutant, [U3C, U8C], which alters different residues, but should have a similar effect on the secondary structure to that of [G4A, G9A]. Second, I have constructed a plasmid for integration of SRP RNA mutants into the chromosome. After the [G4C] mutant allele has been integrated, suppressor analysis and synthetic lethality techniques can be employed to identify fission yeast homologs of the mammalian SRP9p and SRP14p proteins.

MATERIALS AND METHODS

Materials

T4 DNA ligase and DNA polymerase I (Klenow fragment) were purchased from BRL. Restriction enzymes were purchased from either New England Biolabs or BRL. T7 DNA polymerase (Sequenase, version 2.0), and other reagents for DNA sequencing were bought from United States Biochemicals. Oligonucleotide primers used for DNA sequencing reactions were synthesized at the University of Illinois Biotechnology Center. Radioactively labeled compounds ([γ-^{32}P] ATP and [α-^{35}S] ATP) were from ICN Pharmaceuticals Inc. and Amersham Life Sciences Inc. Chemicals for supplementing liquid media and plates were from Sigma.

Mini-Preparations of Plasmid DNA

Mini-preparations of plasmid DNA were carried out according to the alkaline lysis protocol as described in Maniatis *et al.* (Maniatis *et al.*, 1989).

Large Scale Plasmid Preparations

Large amounts of plasmid DNA were purified on a 100 µg scale using materials, reagents, and instructions provided by Qiagen Inc.

Double-Stranded DNA Sequencing

The protocol followed for double-stranded DNA sequencing is as follows: 6 µl mini-prep DNA or 1 µl Qiagen prep DNA (approximately 3-4 µg) was mixed with 4 µl 1 M NaOH (or 4 µl 1 M NaOH and 5 µl distilled water for Qiagen prep DNA), and allowed to sit at room temperature for 5 minutes. Then, 2 µl sequencing primer (100 ng/µl) and 5 µl distilled water was added. Next, 3 µl 3 M NaOAc pH 4.5 and 75 µl cold 100% ETOH was added, then the precipitating DNA was placed on ice for 5 minutes. This was then spun in a microcentrifuge at 4°C for 10 minutes, the supernatant aspirated, 100 µl cold 70% ETOH added, and then spun for 1 more minute. The supernatant was aspirated again, and the pelleted DNA was dried in a speed vacuum for 3 minutes and resuspended in 10 µl 1x Sequenase buffer. Next, the following reagents from the U.S.B. sequencing kit were added to the reaction to reach a total volume of 16 µl: 1 µl 100 mM DTT, 2 µl 1x labeling mix, 1 µl [α^{35}S] ATP and 2 µl Sequenase diluted 1:8 in ice cold TE buffer. The reaction was incubated for 5 minutes at room temperature. 2.5 µl termination mix (ddGTP, ddATP, ddTTP, and ddCTP) was aliquotted into microfuge tubes and pre-warmed to 37°C. Then, 3.5 µl of the labeling reaction was added to each termination mix and incubated at 37°C between 5 and 30 minutes. 4 µl stop solution was added and the mixture heated to 100°C for 2 minutes before the samples were loaded on a 6% polyacrylamide gel.

Restriction Enzyme Digestion

Restriction enzyme digestion was used for the following purposes: to confirm the restriction maps of plasmids used for subcloning, to digest DNA for the actual subclonings, to digest DNA for integration, and to confirm that the plasmids recovered from mini-prep DNA ligation mixtures were transformed into *E. coli* were actually the expected plasmids.

Analytical restriction digests were generally performed in 10 µl volumes with 0.5-1.0 µg plasmid DNA; (10 µg was used for subcloning and 100 µg for isolating the integrant fragment). Each reaction also contained 0.1 volume 10x reaction buffer of the correct salt strength and composition, 1-10 units of enzyme (depending on the application) and water to volume. The digests were incubated at 37°C for 1-2 hours and electrophoresed on gels ranging from 0.7%-2.0% agarose, depending on the application.

In-Gel Ligation Reactions

The procedure used to subclone the constructed plasmids of pWEC.INT Mark2, pWEC.INT Mark2 G4C, and pWEC4.2U is as follows: The plasmids that were to be vectors and the plasmids that contained the inserts were digested with the appropriate enzymes in 20 µl volumes for 1 hour at 37°C. Then, 4 µl of loading dye was added to the digests and 12 µl of the mixture was loaded on a low melting agarose gel containing TAE buffer and 3 µl of 4 mg/ml

ethidium bromide. 100 volts was applied to the gel, and the samples were run until the DNA bands of interest were well separated. The bands to be subcloned were excised from the gel and placed in separate eppendorf tubes. They were heated at 70°C for 10 minutes, vortexed, spun down and heated again at 70°C for 1 minute. The vector and insert were mixed in a 1:2 vector to insert ratio in a total volume of 10 µl (in distilled water). The sample was then mixed, and heated to 70°C for 1 minute, with this procedure repeated once. The heated mixture was then placed at 37°C to cool down. Then 10 µl of a master mix (1 µl 200 mM DTT, 1 µl 10 mM ATP, 2 µl 500 mM Tris buffer pH 7.6, 1 µl T4 ligase, 1 µl 100 mM MgCl, and 4 µl distilled water) was added to the sample and mixed. The sample was then incubated overnight at 16°C. The next day, the agarose containing the DNA was melted at 70°C for 10 minutes, mixed, spun and heated to 70°C for 1 minute. The subcloned DNA was then transformed into *E. coli* by electroporation and plated out on 2XYT + ampicillin. The resulting colonies were miniprepped, and analyzed by restriction digestion.

Hydroxyapatite/Gel Filtration Purification of the G4C/*LEU2* fragment

In order for the G4C mutation of SRP RNA to be integrated into *S. pombe*, pWEC.INT Mark 2 G4C was cut with BamHI and PstI, the 4.25 kb fragment with the mutant SRP gene and *LEU2* marker was

isolated, and then transformed into *S. pombe* (see Lithium Acetate Transformation).

The protocol used is as follows: 100 µg of Qiagen purified pWEC.INT Mark 2 G4C plasmid DNA was digested with BamHI and PstI to generate a 4.25 kb fragment. The DNA was then precipitated and electrophoresed on a large 0.7% agarose gel. The 4.25 kb fragment was excised from that gel and then embedded into another large 0.7% agarose gel. Buffer was then added, and a slurry of hydroxyapatite (1.5 ml hydroxyapatite in 1.5 ml 1X TBE Ethidium Bromide) was added to the well of the gel. The gel was then electrophoresed at 40 V for 7 hours for the DNA to run into the HAP. When the DNA moved completely into the HAP, the voltage was turned off, and the 2 ml of HAP/DNA was drawn off with a Pipetman P-1000.

The DNA was eluted from the HAP and desalted through a Bio-gel P60 column according to the following protocol: Bio-gel P60 was equilibrated with TE/EtBr and heated to drive off excess air. 10 ml of gel slurry was then poured into a Bio-Rad poly-prep chromatography column and allowed to settle. After 1 column volume of TE/EtBr had been run through, the HAP/DNA was applied to the top of the column, and the buffer drained to the top of the HAP/DNA. The DNA was then eluted from the HAP using 0.5 M KH_2PO_4 (pH 6.5). Fractions were collected into sterile eppendorf tubes by following the progression of the DNA with UV light. The

DNA was then precipitated and resuspended in TE. The G4C *SRP7* + *LEU2* fragment was then transformed into RM2a (*SRP7*:ura4, *SRP7*) strain of *S. pombe*.

Lithium Acetate Transformation

The pWEC.INT Mark2 G4C and pWEC4.2Xho U3C, U8C mutants were transformed into the *S. pombe* strain RM2a, which is heterozygous for deletion of the *SRP7* gene (Brennwald *et al.*, 1988). The transformations were done according to the following method: cells were grown to a density between 0.2 x 10^7 and 0.7 x 10^7 cells/ml in EMM2 + adenine + leucine containing media (Mitchison, 1970). The cells were then spun at 3K for 5 minutes at room temperature, then resuspended in 20 ml total LTE (0.1 M LiOAc, 10 mM Tris-HCl (pH 7.4), and 1 mM EDTA). They were spun again at 3K for 5 minutes at room temperature, resuspended at a concentration of 5 x 10^8 cells/ml in LTE (1 ml), and incubated at 30°C for 30 minutes, shaking slowly in a water bath. The cells were then split into 150 µl aliquots. 1 µg of Qiagen prep DNA or 5 µg of HAP prep DNA plus 350 µl PTE (50% PEG-3500, 10 mM Tris-HCl (pH 7.4), and 1 mM EDTA) were added to the sample aliquots. No DNA was added to one aliquot as a negative DNA control. The samples were mixed briefly and then incubated at 30°C for 30 minutes in a shaking water bath, and then heated at 42°C for 20 minutes. Following the heat shock, 500 µl of EMM2 plus adenine + uracil were added to each.

mixed gently, spun for 15 seconds in a microfuge, and resuspended in 150 µl of EMM2 plus adenine + uracil. The mutant to be transformed in as a circle, pWEC4.2Xho U3C, U8C, was then plated onto EMM2 + adenine + uracil (100 µg/ml) plates and incubated at 30°C. Transformants, which are diploids containing the knockout (ura$^+$) and the mutant plasmid pWEC4.2Xho U3C, U8C (leu$^+$) could be seen within 3-4 days.

The mutant which was to be integrated into the chromosome of *S. pombe*, the pWEC.INT Mark 2 G4C Bam HI Pst I fragment containing the mutant *SRP7* gene plus a *LEU2* marker gene, was not plated after resuspension in 150 µl EMM2 plus adenine + uracil. Instead, the sample (and a no DNA control) was spun in a microfuge for 10 seconds and then resuspended in 10 ml EMM2 + adenine + uracil. The cells were grown at 30°C with shaking for 24 hours. 100 µl of this culture was plated onto EMM2 + adenine + uracil + 5-fluoroorotic acid (5-FOA) in order to select for replacement of the *SRP7* gene disrupted with *URA4*, by the G4C mutant *SRP7* gene with a flanking leucine marker. The remainder of the 10 ml sample was then spun at 3K for 5 minutes at room temperature and resuspended in 150 µl EMM2 + adenine and uracil. 10 µl and 100 µl of this was then plated out onto EMM2 + adenine + uracil + 5-fluoroorotic acid and then incubated at 30°C. Colonies were visible after 3-4 days.

Random Spore Analysis and Complementation Assay

Once leu+, ura+ transformants of the U3C, U8C mutation that had been plated on EMM2 + adenine grew to a suitable size, sporulation competent (h^+/h^{90}) derivatives identified, located by iodine staining (Gutz *et al.*, 1974). A sporulating colony was transferred into 1 ml distilled water with 5 µl glusulase added to remove spore walls. This mixture was incubated overnight. The cells were then examined under a microscope and counted. This mixture was then spread onto EMM2 + adenine + uracil plates at 1000 and 10,000 spores per plate and incubated at 30°C for 3-4 days. The resulting colonies were streaked onto EMM2 + adenine + uracil in an orderly grid fashion for replica plating. After 2-3 days of growth at 30°C, these colonies were then replica plated onto EMM2 + adenine to see if the U3C, U8C mutation would complement the *SRP7*:ura4 disruption allele (see Figure 5).

Phenotypic Analysis

The leu+, ura+ haploid colonies that contained the disrupted *SRP7*:ura4 gene and the U3C, U8C mutant gene were tested for cold sensitivity by incubation at 18°C, heat sensitivity by incubation at 37°C, and impaired growth at 37°C on media supplemented with 25% glycerol (designated as an Osmotically Temperature Sensitive (OTS) phenotype; Liao *et al.*, 1989).

Construction of pWEC.INT Mark2

In order to begin construction of a targeting vector that was suitable to integrate the conditional allele, the plasmid pTZ19 was digested with Hind III, the overhanging end filled in with the Klenow fragment of DNA polymerase, and religated. Both plasmids, pTZ19 (HindIII) and pWEC.INT were digested with BamHI and PstI. The 4.25 kb fragment from pWEC.INT was ligated into the 2.84 pTZ19 (HindIII) backbone after excising both fragments from a 0.7% NuSieve gel (see Figure 6).

Construction of pWEC.INT Mark2 G4C

In order to create a unique HindIII site required for easy subcloning of the mutant *SRP7* gene, the mutant plasmid pWEC4.2 XhoI G4C and the targeting vector pWEC.INT Mark 2 were digested with Hind III and XhoI, the fragments were excised from a 1% NuSieve gel, and the 0.48 kb fragment of pWEC4.2 XhoI G4C was ligated into the 6.61 kb backbone fragment of pWEC.INT Mark 2 (see Figure 7).

Construction of pWEC4.2U

In order to conduct synthetic lethality experiments on the G4C mutation in *S. pombe* , a suitable vector with a *URA4* yeast marker instead of a *LEU2* marker is required. To create this plasmid, the plasmids pUTZ4 and pWEC4.2 were digested with HindIII, the

fragments separated and excised from a 1.0% NuSieve gel, and the 1.71 kb fragment from pUTZ4 was ligated into the 5.58 kb fragment from the pWEC4.2 backbone (see Figure 8).

RESULTS AND DISCUSSION

Mutational Analysis of the SRP9/14p Binding Site

One of the alleles created by random mutagenesis (Liao *et al.*, 1992) was the [G4A, G9A] containing one mutation in the pentanucleotide hairpin loop near the 5' end of the SRP RNA and a second substitution in the 3' side of the stem (see Figure 1). As noted in the Introduction, this region of *S. pombe* SRP RNA corresponds to the SRP9/14 heterodimeric protein binding site of the mammalian SRP particle. Growth of the [G4A, G9A] strain was found to be severely impaired at 37°C on 25% glycerol containing media (designated a severe OTS phenotype, Liao *et al.*, 1989) when the mutant gene was expressed from an extrachromosomal plasmid. To ascertain which nucleotide substitution was responsible for the growth defect, the single mutations [G4A] and [G9A] were constructed and characterized (Liao *et al*, 1992). Surprisingly, both alleles allowed growth at wild type rates. Fortunately, at the same time, the mutation [G4C] had been created. This mutant does manifest the same conditional lethality as the original double mutant. These observations led to a model in which both the identity of the nucleotides and the structure of the RNA surrounding the seven nucleotide conserved sequence are proposed to be critical for binding of a fission yeast SRP9/14p homolog.

I have carried out experiments aimed at further refining this model by determining the phenotypes of additional mutants. The first allele I examined was [U3C, U8C], originally constructed by Anne Chiang, a former undergraduate in the lab. The predicted structure of the 5' end of this RNA is similar to that of the [G4A,G9A] mutant (see Figures 4, 5 and 9); however, the identity of the nucleotides is changed. The double mutant creates a Watson-Crick (WC) base pair between positions 4 and 8, and changes a U-G wobble pair to an A-U WC base pair. Because position 4 remains unchanged, the consequences of these mutations should further separate the contributions of structure and nucleotide identity, since the model stipulates that the nucleotide at position 4 must be a single stranded purine to allow wild type growth to occur.

It was predicted that this mutant would, like [G4A, G9A], have a severe OTS phenotype due to the increased stability of the secondary structure created by the introduction of two G-C base pairs in place of a G-U and two unpaired nucleotides. The [U3C, U8C] mutant was able to complement the null allele *SRP7*::ura4 knockout in the strain RM2a (see Figure 5), since the cells were viable with the mutant as the only copy of the *SRP7* gene. As expected, the mutant had an OTS defect, but was still able to grow slowly on 25% glycerol plates at 37°C (by definition, a mild OTS defect; see Liao *et al.*, 1989). One explanation for the less severe phenotype relative to [G4C] is

that the increased stability does not contribute as much to the conditional phenotype as the identity of the nucleotide at position 4.

I also attempted to make two other mutations, [U5G] and [U5C], since their phenotypes should distinguish the importance of the identity of the highly conserved position 5 nucleotide in the putative SRP9/14 binding site (see Figure 9). It is reasonable to predict that the transition from U to C may confer a less severe conditional growth defect, since the wild type nucleotide is also a pyrimidine and the mutation should not result in additional base pairing. The transversion from U to G is analogous to the [G4C] mutation. Thus, if the [U5G] mutation confers a severe OTS phenotype, we would conclude that position 5 is also important for SRP9/14p recognition of the RNA.

Construction of the Integration Plasmid pWEC.INT Mark2 G4C

In addition to elucidating the structure and identity requirements within the putative SRP 9/14p binding site of *S. pombe* SRP RNA, I have been working toward integrating one of the 5' end conditional mutants and conducting suppressor analysis and synthetic lethality experiments to identify fission yeast homologs of SRP9p and SRP14p.

In order to integrate the [G4C] mutant into the *S. pombe* genome, a suitable vector needed to be constructed. The reason it was necessary to subclone the mutant *SRP7* gene into a different

vector was two-fold. First, pWEC4.2XhoG4C is a replicating plasmid containing *ARS1*, an autonomously replicating segment necessary for the plasmid to replicate extrachromosomally in *S. pombe*. Such a construct allows complementation assays such as the one depicted in Figure 5 to be employed to ascertain whether the mutant allele can support viability. Second, it was necessary to introduce an *S. pombe* selectable marker into the flanking sequence of the *SRP7* gene such that both genes could be excised on a single fragment.

The plasmid pWEC.INT, carrying a wild type copy of the *SRP7* gene and a *LEU2* selectable marker, was constructed by Anne Chiang, a former undergraduate student in the lab. This plasmid was designed to replace an earlier [G4C] integrative plasmid, pWEC6.7SC4, which did not contain enough sequence upstream of the *SRP7* gene to allow integration of the present mutant into the genome. Only 100 base pairs upstream of the *SRP7* gene are present in pWEC6.7SC4, while at least 500 base pairs both 5' and 3' of the gene are required for high frequency recombination in *S. pombe*.

However, it was necessary to further modify the pWEC.INT plasmid because it contained two HindIII sites. A unique HindIII site was required to allow subcloning of the [G4C] mutant *SRP7* gene, and to allow use of this shuttle vector for future subcloning of alleles carrying mutations not just at the 5' end, but anywhere in the *SRP7* gene. To create pWEC.INT Mark2, the 4.25 kb BamHI-PstI fragment from pWEC.INT was ligated into the 2.86 kb backbone of pTZ19

(HindIII) (in which the HindIII site had been deleted; see Figure 6). The presence of a unique HindIII site allows HindIII-XhoI fragments from plasmids containing mutant *SRP7* genes to be used to replace the wild type gene. For the [G4C] allele, the source plasmid was pWEC4.2XhoG4C, which has HindIII and XhoI sites immediately surrounding the mutant *SRP7* gene (Figure 7). The resulting 0.48 kb HindIII-XhoI fragment could be ligated into the 6.61 kb backbone to form the mutant integrative plasmid (see Figure 7). The pWEC.INT Mark2 G4C construction was then used to prepare large quantities of a linear fragment to be used for integrating the [G4C] allele into the *S. pombe* genome.

Integration of the pWEC.INT Mark2 G4C fragment into the *S. pombe* genome

A BamHI-PstI 4.25 kb fragment from pWEC.INT Mark2 G4C, containing the mutant gene and a *LEU2* marker, was isolated and transformed into *S. pombe*. Several fates are possible for the linear fragment: 1) the fragment could circularize and exist as an extrachromosomal element; 2) the fragment could integrate at an incorrect chromosomal locus; or 3) the fragment could integrate at the correct chromosomal locus.

To eliminate the first possibility, stability tests can be conducted to determine if the fragment is lost under non-selective conditions. The transformed colonies are grown in rich media and

after several dilutions, are plated out on minimal media plus the supplements required to select for the marker carried on the integrative fragment.

The next step is to differentiate between colonies that have the fragment integrated at the correct, versus incorrect, location. This will be determined by performing Southern hybridizations on genomic DNA from stable transformants. This technique utilizes a radioactive DNA probe that is complementary to the locus of interest to probe DNA digested with various restriction enzymes.

Finally, a strain with the correct integration event will be subjected to phenotypic analysis by examination of random spores. For [G4C], we expect that 50% of the colonies will exhibit an OTS phenotype and 50% will be wild type.

After it has been determined that integration occurred at the correct chromosomal locus and that the phenotype is similar to that of the extrachromosomal allele (as has been the case for other integrated *SRP7* alleles; Selinger *et al.*, 1993), suppressor analysis can be conducted. Suppressors are mutations in other genes which overcome the original mutant phenotype. Suppression can occur through a number of mechanisms: the suppressor can be a close structural and/or functional homolog of the original mutant gene; the gene product of the suppressor could physically interact with the gene product of the mutant gene; or increased expression of a suppressor gene product could totally bypass the need for the

mutant gene product (Russell, 1989). There are two types of suppressor analysis that can be used for an integrated mutant. In high copy suppression, the integrant is transformed with a fission yeast DNA library on a high copy plasmid, and then selected for growth under OTS conditions (rich media plus 25% glycerol plates grown at 37°C). The plasmid will be isolated from the surviving colonies and analyzed. In the case of the [G4C] allele, the suppressors might be 1) genes encoding the *SRP7* gene itself; 2) genes encoding SRP9p or SRP14p; or 3) other genes whose products can bypass the SRP pathway. The suppressor genes will be sequenced to identify the protein or RNA responsible for suppression.

For standard suppressor analysis, the integrant is mutagenized and selected for growth as before, and then backcrossed with a wild type strain to check for extragenic or intragenic suppression. The extragenic suppressors will be tested for conditional phenotypes, which would allow their genes to be cloned by complementation.

At this point, I am conducting stability tests to determine whether the mutant *SRP7::LEU2* fragment has been stably integrated into the genome. After stable transformants are isolated, Southern hybridization will be carried out to confirm that the mutant gene was integrated at the correct chromosomal locus and the phenotype will be determined. After these procedures, high copy and standard suppressor analysis will be conducted, as well as synthetic lethality

experiments (see below), to search for extragenic suppressors that could be fission yeast homologs of SRP9/14p.

Construction of pWEC4.2U

Synthetic lethality experiments are another way of searching for genes that are not in the same pathway as the mutation of interest. A synthetic phenotype, in general, is a more severe, often lethal, phenotype caused by two different conditional alleles from two different genes (Huffaker *et al.*, 1987). Since the [G4C] mutation confers a conditional (OTS) phenotype, a haploid of *S. pombe* containing this mutation can be transformed with a wild type *SRP7* gene on pWEC4.2U. The cells can then be mutagenized, and the resulting colonies replica plated onto EMM2 + adenine + 5-FOA (see Lithium Acetate Transformation) to select for plasmid loss. Colonies that grow slowly or not at all on 5-FOA, which cures the plasmid carrying the wild type gene, are likely to harbor synthetic lethal mutations. These strains will be isolated from the original plate and crossed with a wild type strain and sporulated. The mutation responsible for the synthetic lethality will have a phenotype of its own, which can be exploited to clone the gene to which it maps.

In conclusion, I have shown that the [U3C, U8C] allele carrying mutations at the 5' end of *S. pombe* SRP RNA exhibits a mild OTS phenotype. This demonstrates that the identity of the position 4 nucleotide may be more important than secondary structure for *S.*

pombe SRP9/14p binding. Additional mutational analysis of the SRP9/14p binding site will be conducted in the future, as well as suppressor analysis and synthetic lethality experiments to identify fission yeast homologs of the SRP9p and SRP14p polypeptides.

REFERENCES

Beach, D., Piper, M., and Nurse, P. (1982) *Mol. Gen. Genet.* 187: 326-329.

Brennwald, P., Liao, X., Holm, K., Porter, G., and Wise, J.A. (1988) *Mol. Cell Biol.* 8: 1580-1590.

Gutz, H., Heslot, H., Leupold, U., and Loprieno, N. (1974) in *Handbook of Genetics*, ed. King, R. C., Plenum Publishing Corp., New York.

Hann, B. C. and Walter, P. (1991) *Cell* 67:131-144.

Huffaker, T. C., Hoyt, M. A., and Botstein, D. (1987) *Ann. Rev. Genet.* 21: 259-84

Krieg, U. C., Walter, P. and Johnson, A. E. (1986) *Proc. Natl. Acad. Sci. USA* 83: 8604-8608.

Kurzchalia, T. V., Wiedmann, M., Girshovich, A. S., Bochkareva, E.S., Bielka, H. and Rapoport, T. (1986) *Nature* 320: 634-636.

Liao, X., Brennwald, P., and Wise, J. A. (1989) *Proc. Natl. Acad. Sci. USA* 86: 4137-4141.

Liao, X., Selinger, D., Althoff, S., Chiang, A., Hamilton, D., Ma, M., and Wise, J. A. (1992) *Nucleic Acids Res.* 20: 1607-1615.

Maniatis, T., Fritsch, E., and Sambrook., J. (1989) *Molecular Cloning: A Laboratory Manual* (2nd Edition). Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Meyer, D. I., Krause, E., and Dobberstein, B. (1982) *Nature* 297: 647-50.

Mitchison, J. M. (1970) *Meth. cell. Physiol.* 4: 131-154.

Ogg, S. C., Poritz, M. A., Walter, P. (1992) *Molec. Biol. Cell* 3: 895-911.

Poritz, M. A., Siegel, V., Hansen, W. B., and Walter, P. (1988) *Proc. Natl. Acad. Sci. USA* 85: 4315-4319.

Ribes, V., Dehoux, P. and Tollervey, D. (1988) *EMBO J.* 7: 231-237.

Ribes, V., Romisch, K., Giner, A., Dobberstein, B., and Tollervey, D. (1990) *Cell* 63: 591-600.

Russell, P. (1989) in Nasim, A., Young, P., and Johnson, B. F. (ed.) *Molecular Biology of the Fission Yeast*. Academic Press Inc, New York, p.247-248.

Selinger, D. A., Brennwald, P. J., Liao, X., and Wise, J. A. (1993) *Mol. Cell. Biol.* 13: 1353-1362.

Siegel, V., and Walter, P. (1985) *J. Cell Biol.* 100: 1913-21.

Siegel, V., and Walter, P. (1986) *Nature* 320: 81-84.

Siegel, V., and Walter, P. (1988a) *Cell* 52: 39-49.

Siegel, V. and Walter, P. (1988b) *Proc. Natl. Acad. Sci. USA* 85: 1801-1805.

Strub, K., Barnett-Moss, J., and Walter, P. (1991) *Mol. Cell Biol.* 11: 3949-3959.

Tajima, S., Lauffer, L., Rath, V. L., and Walter, P. (1986) *J. Cell Bio.* 103: 1167-1178.

Walter, P., and Blobel, G. (1980) *Proc. Nat. Acad. Sci. USA* 77: 7112-7116.

Walter, P., and Blobel, G. (1981) *J. Cell Biol.* 91: 557-561.

Walter, P., and Blobel, G. (1982) *Nature* 299: 691-98.

Walter, P., and Lingappa, V.R. (1986) *Annu. Rev. Cell Biol.* 2: 499-516.

Wiedmann, M., Kurzchalia, T. V., Hartmann, E., and Rapoport, T. A. (1987) *Nature* 328: 830-833.

Wolin, S.L., and Walter, P. (1989) *J. Cell Biol.* 109: 2615-2622.

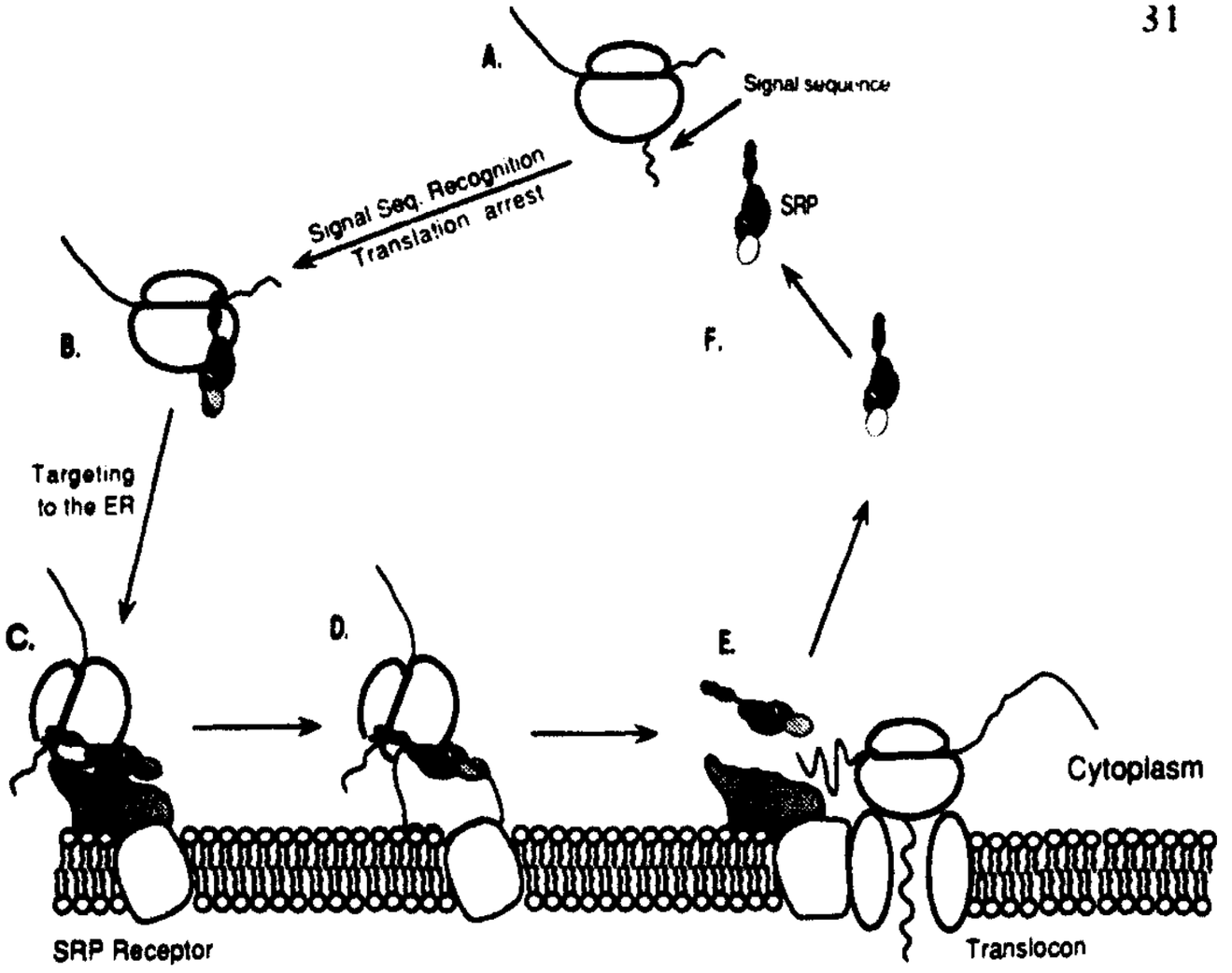

Figure 1. A model of the SRP cycle (modified from doctoral thesis of David Selinger) (A) a ribosome translating a secretory protein is recognized by SRP, (B) SRP binds the signal sequence and translation is halted, (C) the complex is targeted to the ER membrane where SRP interacts with its receptor, (D) the SRP-receptor complex changes conformation (E) the receptor complex comes in contact with the translocon and the SRP/nascent chain chain complex is released, (F) SRP cycles back into the cytosol.

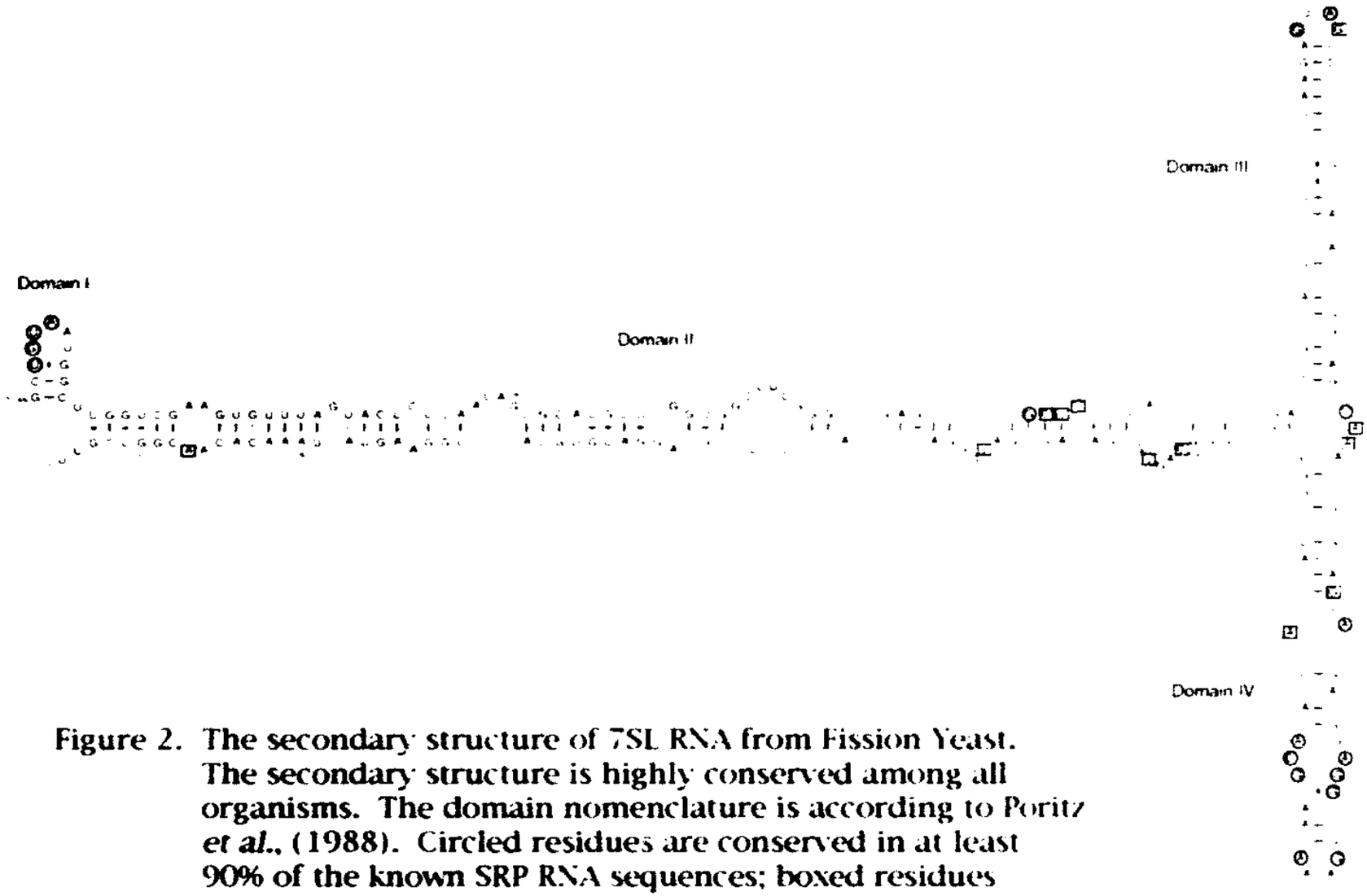

Figure 2. The secondary structure of 7SL RNA from Fission Yeast. The secondary structure is highly conserved among all organisms. The domain nomenclature is according to Poritz *et al.*, (1988). Circled residues are conserved in at least 90% of the known SRP RNA sequences; boxed residues denote positions in which either a purine or pyrimidine base is found in all SRP RNAs.

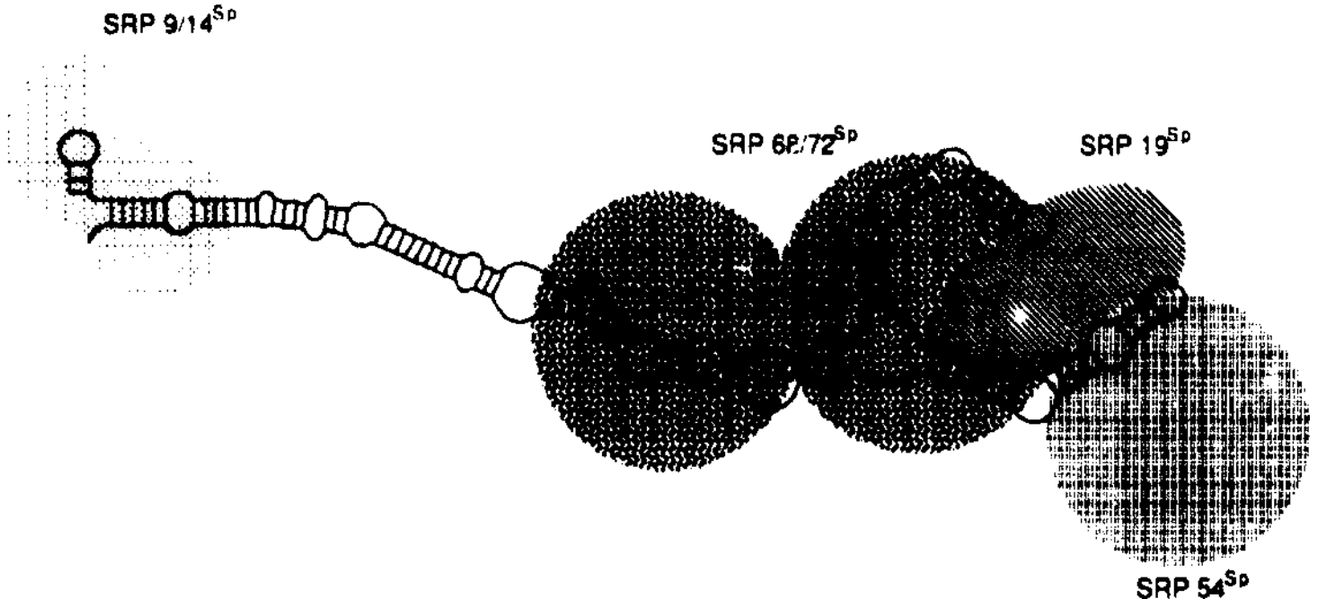

Figure 3. Model of fission yeast SRP (structure adapted from Liao *et al.*, 1992).

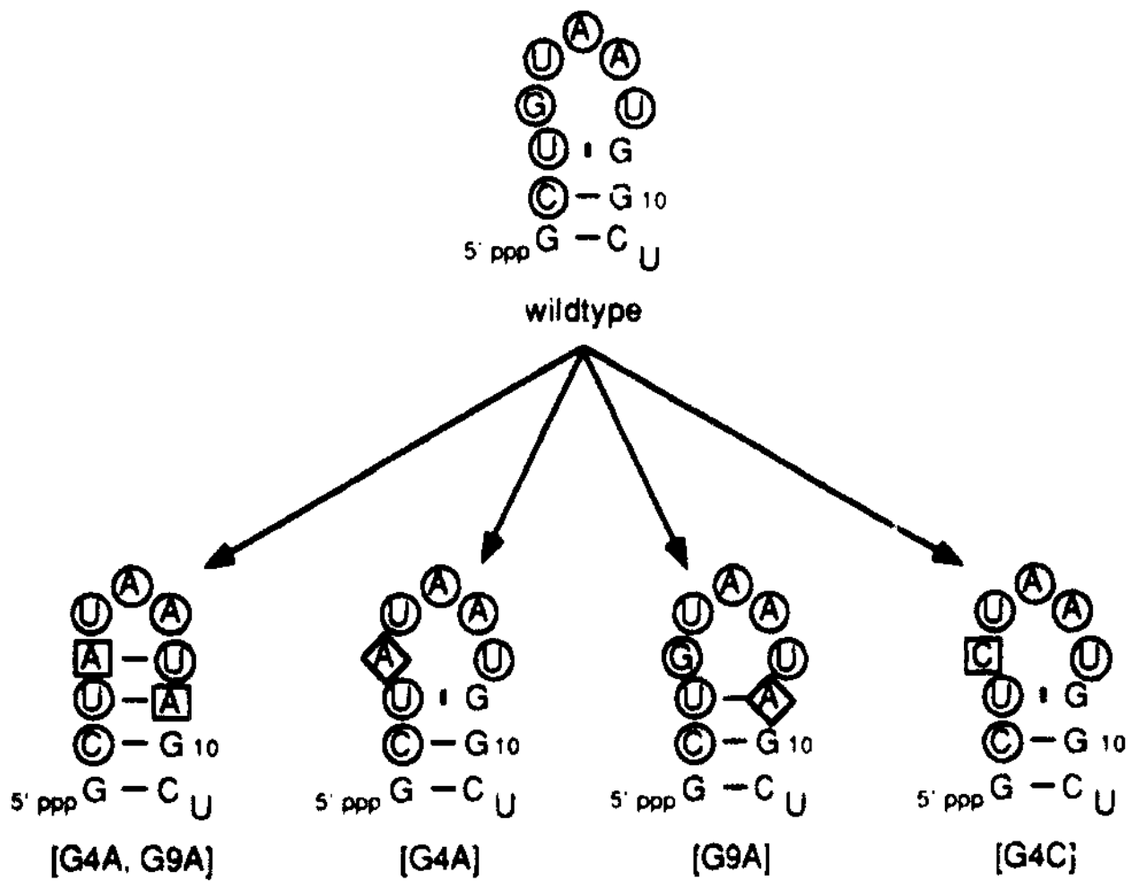

Figure 4. Effects of mutations in the SRP9/14 protein binding site. Conserved residues corresponding to those protected from hydroxyl radical cleavage in the human RNA (Strub *et al.*, 1991) are circled. Residues substituted in mutant alleles which confer conditional growth defects are boxed, whereas those which allow wild-type growth are enclosed by a diamond.

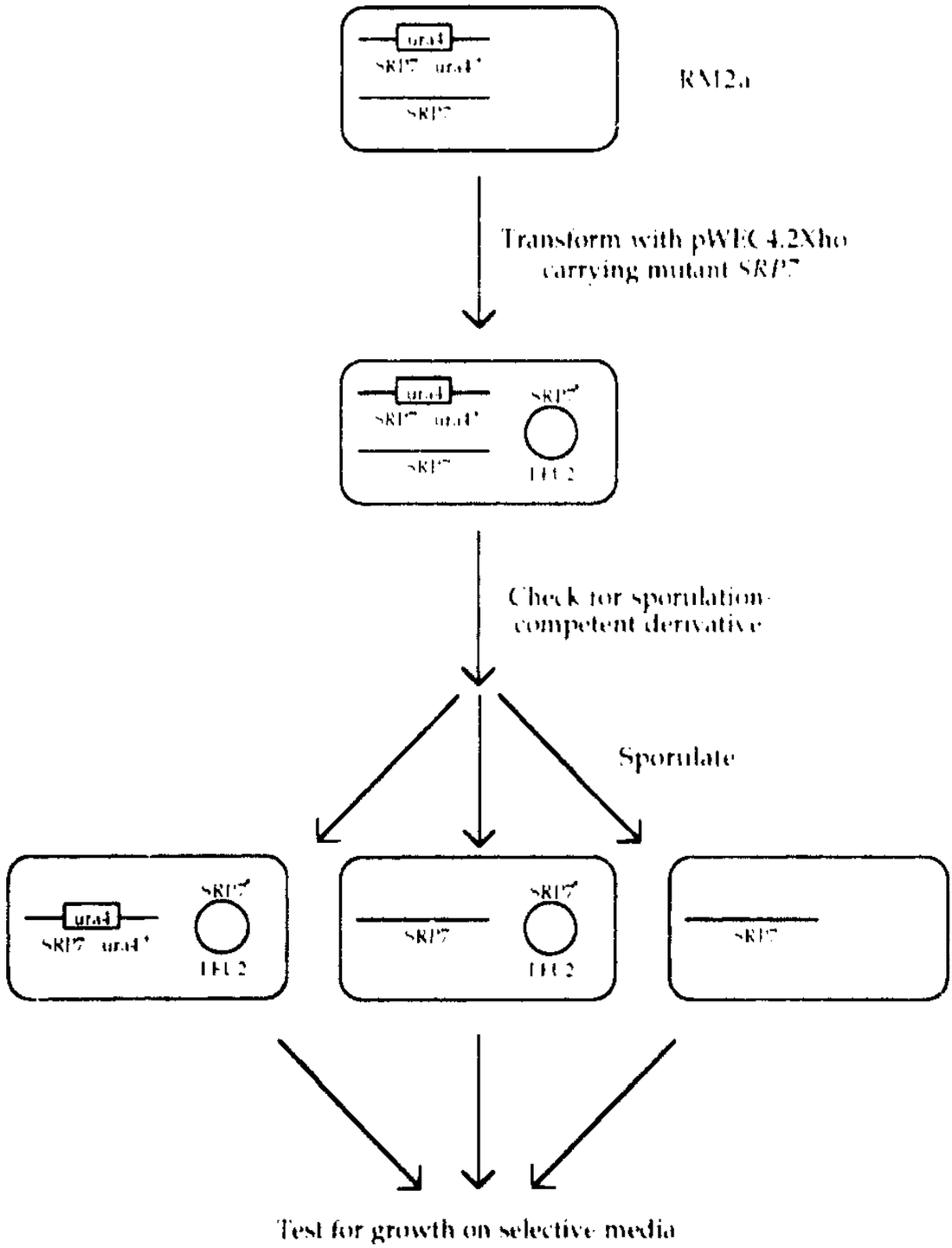

Figure 5. Random spore complementation test for *SRP7* function of the [U3C, U8C] mutant allele.

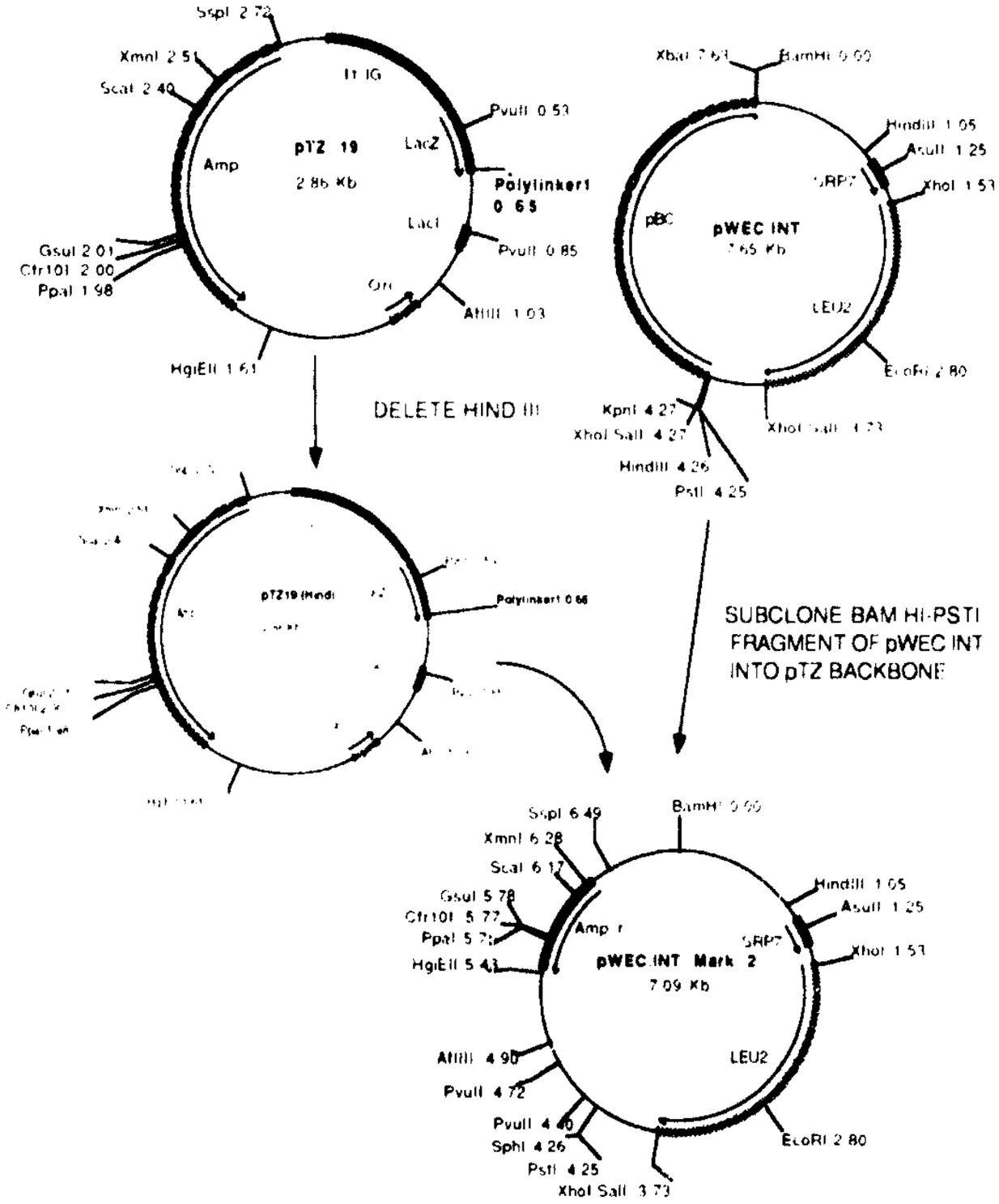

Figure 6. Construction of the plasmid pWEC.INT Mark2.

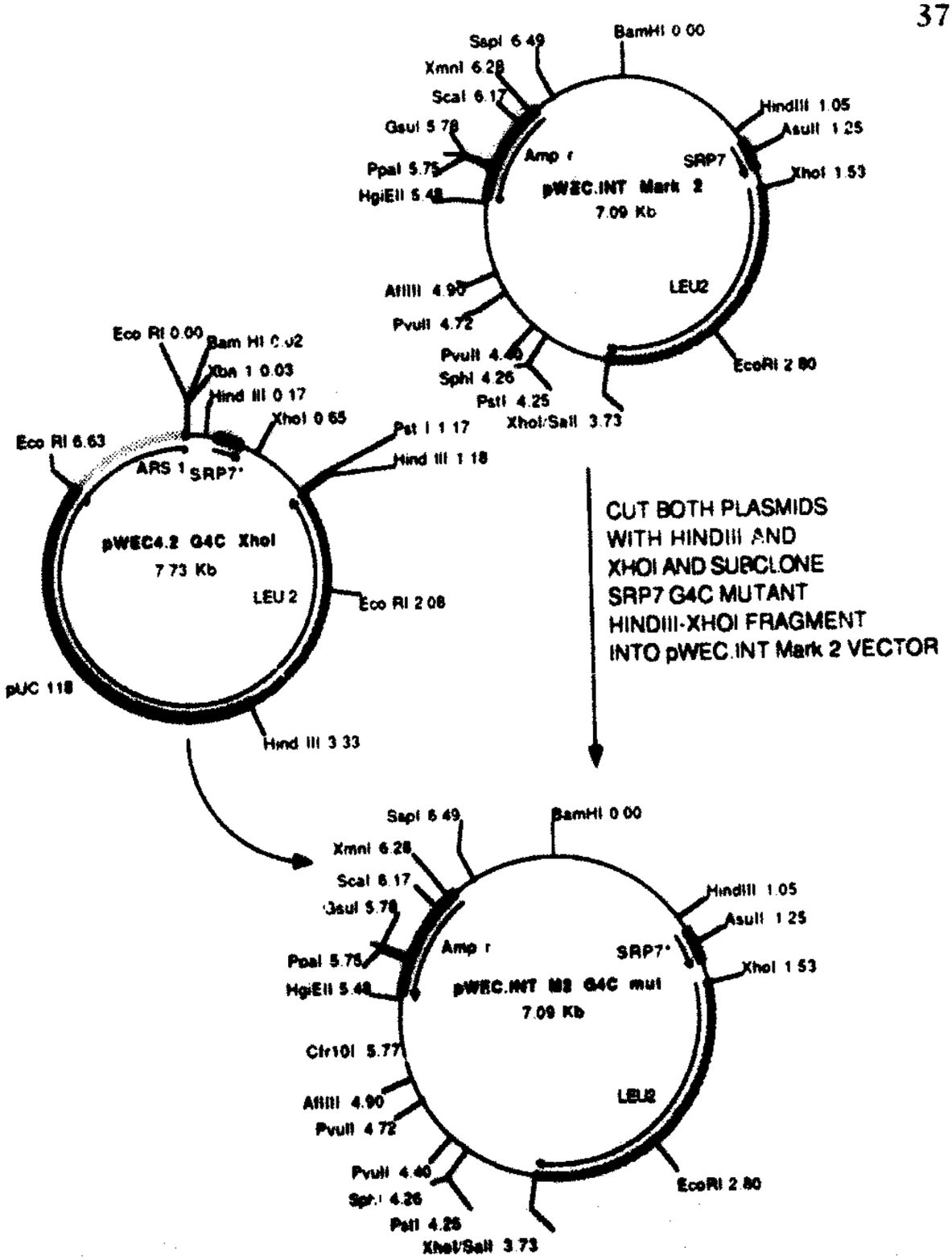

Figure 7. Construction of the plasmid pWEC.INT Mark2 G4C.

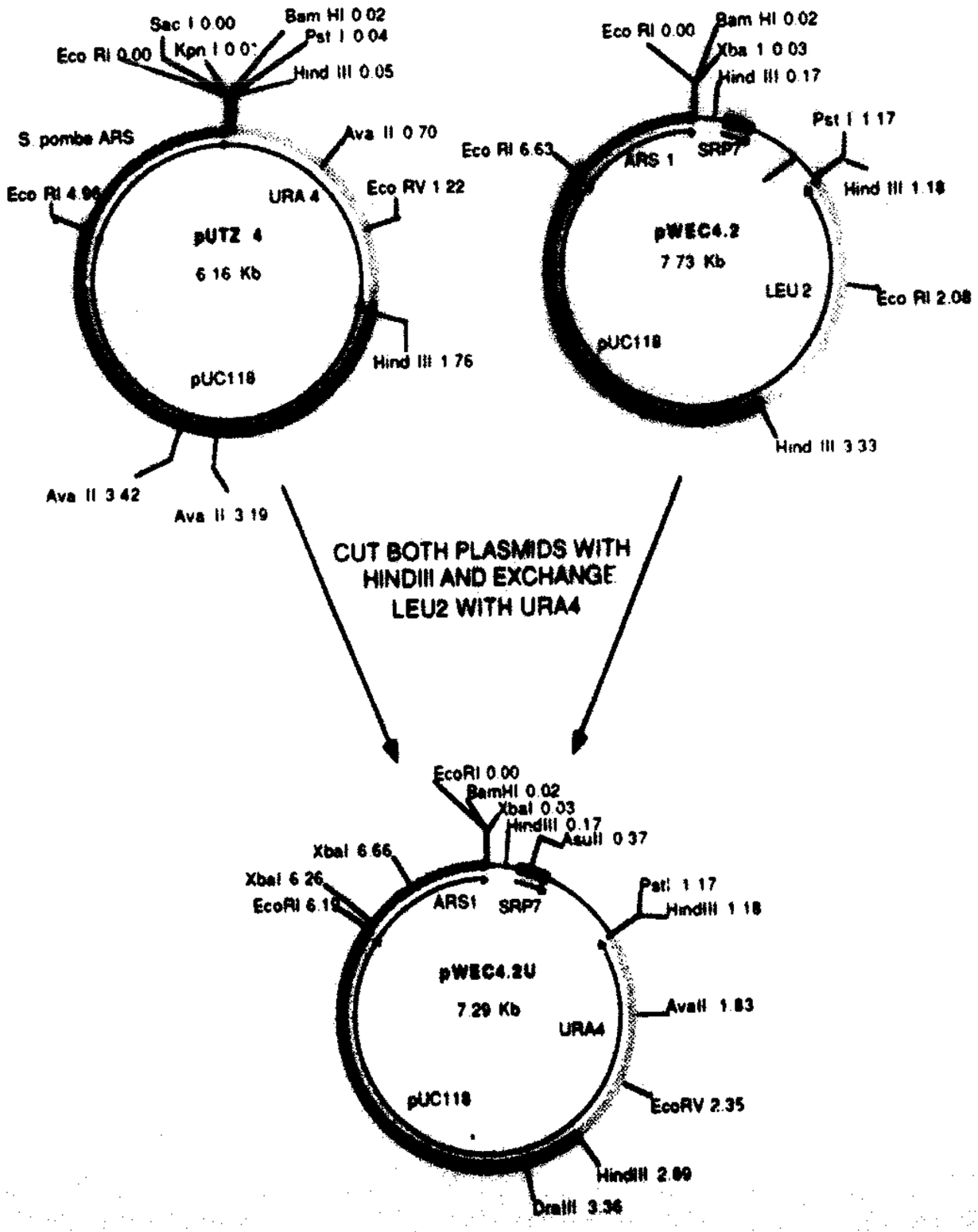

Figure 8. Construction of the plasmid pWEC4.2U.

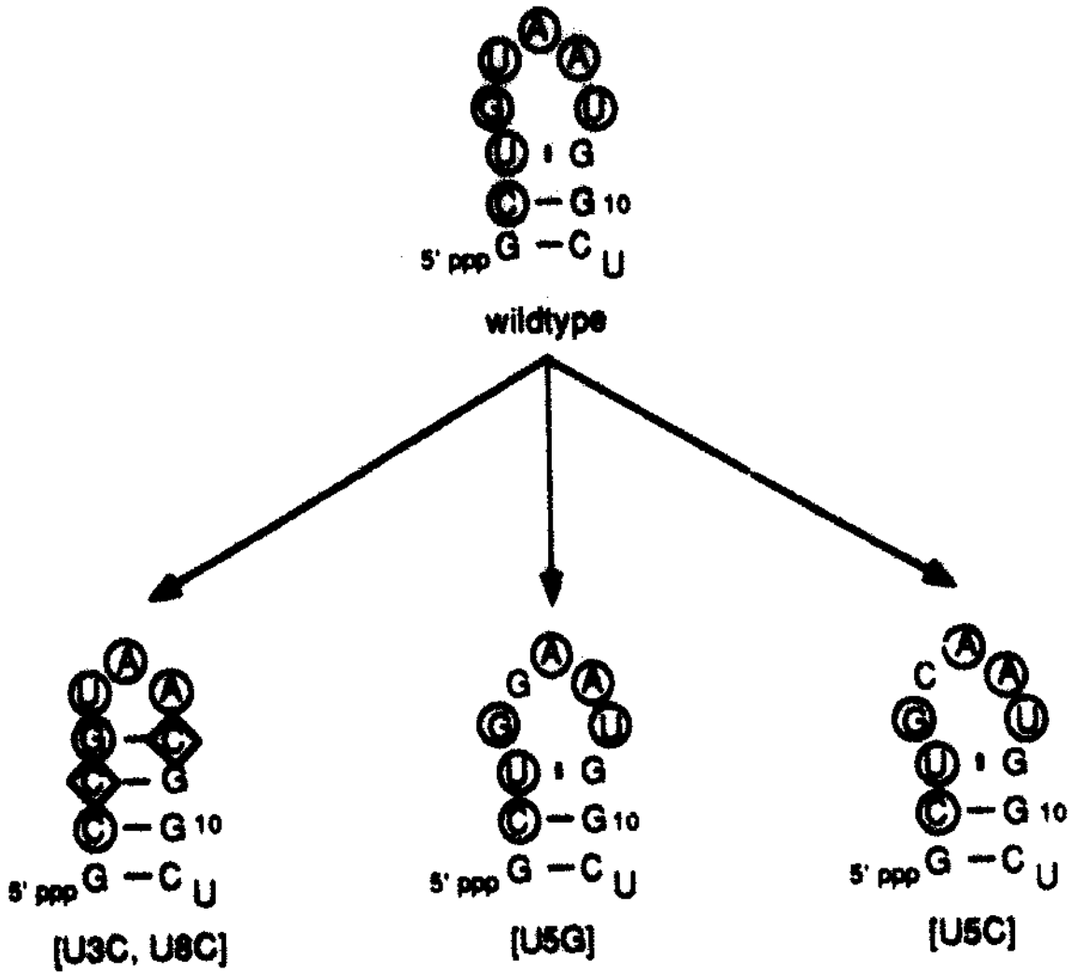

Figure 9. Predicted secondary structure of mutations [U3C, U8C], [U5G],and [U5C]. Conserved residues corresponding to those protected from hydroxyl radical cleavage in the human RNA (Strub *et al.*, 1991) are circled.