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

SCOTT WALTER STEVENS

ENTITLED CLONING, DISRUPTION, AND MUTATIONAL ANALYSIS OF THE SRP54 PROTEIN HOMOLOGUE IN SCHIZOSACCHAROMYCES POMBE

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

Instructor in Charge

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HEAD OF DEPARTMENT OF BIOCHEMISTRY

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CLONING, DISRUPTION, AND MUTATIONAL ANALYSIS OF THE SRP54 PROTEIN HOMOLOGUE IN SCHIZOSACCHAROMYCES POMBE

BY

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INTRODUCTION

Protein secretion is a necessary function in the life of a cell. Among the variety of different fates of a secreted protein in the cell are: export into the extracellular space, integration into a variety of membranes, and incorporation into organelles such as the lysosome. Secretion is accomplished by one of two mechanisms, the first being the post-translational secretory pathway. In this pathway, the completely synthesized secretory protein is targeted to its destination and translocated. The second mechanism is the co-translational secretory pathway. In this pathway, the function of signal recognition particle (SRP) is to target ribosomes translating pre-secretory proteins to the endoplasmic recticulum (ER) membrane.

SRP in mammals is a ribonucleoprotein particle that has been shown to function in protein translocation across the ER membrane (Walter and Blobel, 1980, 1982). The 11S particle consists of a 7SL RNA component as well as six polypeptide components of M_r 72,000, 68,000, 54,000, 19,000, 14,000, and 9,000 daltons; designated SRP72, SRP68, SRP54, SRP19, SRP14 and SRP9 respectively. Homologues of mammalian SRP have been found and studied in plant (Campos et al.,

1988), and in genetically tractable systems such as bacteria (Poritz et al., 1988a, 1990; Ribes et al., 1990), and fungi (Brennwald et al., 1988; Poritz et al., 1988b).

The mechanism of SRP action, which was first postulated by Peter Walter and Günter Blobel (Walter and Blobel, 1980), is as follows. An mRNA coding for a pre-secretory protein begins to be translated by a ribosome. After the signal sequence—which consists of 10-20 hydrophobic amino acids—emerges, SRP associates with the ribosome-peptide moiety and momentarily arrests translation (Walter and Blobel, 1980). This affinity exists only while the ribosome is translating the mRNA. During the translation arrest, the SRP complex "docks" on the ER via the SRP receptor (Gilmore and Blobel, 1983; Savitz and Meyer, 1990). The nascent polypeptide is completed by the ribosome as it is translocated by an unknown mechanism through the ER membrane.

In the fission yeast Schizosaccharomyces pombe (S. pombe), SRP is also an 11S complex and shares many similar features with mammalian SRP (Brennwald et al., 1988; Selinger et al., 1991). The 7SL RNA component is similar in size to and can be folded into a secondary structure virtually identical to that of human 7SL RNA (Zweib, 1985; Brennwald et al., 1988). The S. pombe protein

homologues are very similar in size to the mammalian SRP proteins. In S. pombe, SRP72 is 76 kD, SRP68 is 63 kD, SRP54 is 57 kD ($SRP54^{Sp}$), SRP19 is 23 kD, SRP14 is 15 kD, and SRP9 is 10 kD (Selinger et al., 1991). In mammals, these six proteins consist of two monomers and two heterodimers which each serve a specific function. Removal of the Alu domain (Figure 1), or the 9 kD and 14 kD proteins which bind to it, disrupts translation arrest (Siegel and Walter, 1985). By chemical modification, the 68 kD/72 kD heterodimer has been shown to function in translocating the protein through the ER membrane (Siegel and Walter, 1988a, 1988b). The 19 kD and 54 kD proteins associate with 7SL RNA in domains III and IV (Figure 1), but serve different functions. Under most conditions, the 19 kD protein is required for the binding of SRP54 to the particle (Römisch et al., 1990). This suggests that the 19 kD protein may mediate a conformational change in 7SL RNA. SRP54 is involved in the recognition of the signal sequence of the nascent polypeptide as evidenced by photo crosslinking experiments (Kurzchalia et al., 1986; Kreig et al., 1986). SRP54 was originally thought to bind to SRP via SRP19; however, recent data shows that the methionine rich carboxy terminus of SRP54 can be cross-linked to mammalian 7SL RNA

(Römisch et al., 1990; Zopf et al., 1990) and also binds to the E. coli 4.5S RNA (Poritz et al., 1990; Ribes et al., 1990).

Mutants of S. pombe 7SL RNA in the highly conserved domain IV tetraloop have a variety of phenotypes (Liao et al., 1989; Brennwald et al., 1991). Immunopreciptiation experiments show that disruption of the base pair just preceding the tetraloop in domain IV produces a 7SL RNA that does not stably bind to $SRP54^{Sp}$ (Brennwald et al., 1991). This implies that $SRP54^{Sp}$ makes contacts in that region, and that the closing base pair is necessary for optimal binding.

$SRP54^{mam}$ shares extensive protein sequence similarity with its counterparts in fission yeast ($SRP54^{Sp}$), budding yeast ($SRP54^{Sc}$) and E. coli (fifty-four homologue, or ffh) (Hann et al., 1989). E. coli 4.5S RNA shares some sequence homology and a conserved secondary structure with that of the fourth domain of 7SL RNA (reviewed in Poritz et al., 1988a). All homologues of SRP54 can be divided into three domains (Figure 2). The amino terminal domain (N domain) contains a stretch of very highly conserved amino acids, but has not yet been assigned a function. A domain located in the middle of the protein contains a GTP binding motif (G domain) (Römisch et al., 1989; Bernstein et al., 1989), and the carboxyl terminal domain

contains a methionine rich domain (M domain) which has been implicated in the binding of the signal sequence as well as 7SL RNA (Zopf et al., 1990; Römisch et al., 1990). The G domain contains four conserved boxes which are similar to the G-1 through G-4 regions defined by sequence comparisons of ras, EF-Tu and other G proteins; however, these exhibit some potentially significant differences as well (see Figure 3). Deletion mutants of SRP54 that contain only the M domain, as well as the M domain plus various portions of the G domain, are able to bind 7SL RNA in the presence of SRP19 (Römisch et al., 1990; Zopf et al., 1990). Deletion mutants that contain little or no M domain do not bind 7SL RNA.

This thesis presents evidence that SRP54 is an essential component of SRP and that mutations in the G-domain can affect the proper functioning of SRP54.

MATERIALS AND METHODS

Materials: Restriction enzymes KpnI, NheI, XhoI, and SalI were purchased from New England Biolabs; all other restriction endonucleases, T4 DNA ligase, T4 DNA kinase, Klenow polymerase (large fragment), and associated buffers were purchased from Bethesda Research Laboratories. Taq DNA polymerase was purchased from Beckman. [γ-^{32}P]ATP and [α-^{32}P]dCTP were purchased from ICN Pharmaceuticals, Inc. [α-^{35}S]dATP and multiprime DNA labelling system for radioactive DNA labelling were purchased from Amersham Corporation. DNA sequencing was performed using a kit from United States Biochemicals (U.S.B.). T7 DNA polymerase used for mutagenesis reactions and calf intestine alkaline phosphatase were also purchased from U.S.B. Oligonucleotide primers for sequencing and mutagenesis, and XhoI linkers were synthesized at the University of Illinois Biotechnology Center. M13KO7 bacteriophage and bacterial strain BMH71-18mutS were purchased from Promega Corp. Amino acid and nucleotide supplements to media as well as Glusulase were purchased from Sigma. Bactotryptone, yeast extract, and agar were from Difco Labs. Novozym was purchased from Novo Industries.

Analytical PCR: In order to determine whether the gene encoding $SRP54^{Sp}$ was present in the available libraries in our lab, I used the polymerase chain reaction. The λ-DASH library was constructed by Dave Selinger from a Sau3AI partial digest of S. pombe genomic DNA ligated into the BamHI site the λ-DASH vector (Stratagene). Prior to ligation, λ DNA was extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1), once with chloroform:isoamyl alcohol (24:1), and then ethanol precipitated. S. pombe genomic DNA was prepared by Pat Brennwald. The PstI library was constructed from a PstI complete digest of S. pombe genomic DNA inserted into the pUC18 vector. The pFL20 library was constructed from a Sau3AI partial digest of S. pombe genomic DNA ligated into the BamHI site of the vector pFL20 (Lasson and Lacroute, 1983). PCR was performed in 100 µl total volume containing 50 mM KCl, 10 mM Tris HCl pH 8.3, 1.5 mM $MgCl_2$, 150 µM each dNTP, 250 ng each of oligonucleotide primers M1 and M2 (Figure 9) which amplify the 3' 600 nucleotides of $SRP54^{Sp}$, and 500 ng DNA from each library. The samples were denatured at 95°C for 5 minutes before addition of 1 unit of Taq DNA polymerase. The samples were then annealed at 45°C for 5 minutes, and polymerized at 65°C for 2 minutes. Two cycles of denaturation at 92°C for 1 minute, annealing at 50°C for 1

minute and polymerization at 70°C were followed by 27 cycles of denaturation at 92°C for 1 minute, annealing at 55°C for 1 minute and polymerization at 70°C for 2 minutes. Samples were removed and analyzed on an agarose gel.

Screening the λ bank for $SRP54^{Sp}$: Bacterial strain P2-392 was infected with the λ-DASH library. The infected cells were plated out on NZCYM plates (1% casein acid hydrolysate, 0.5% NaCl, 0.5% yeast extract, 0.01% casamino acids, 0.02% $MgSO_4 \cdot 7H_2O$, 1.5% agar) at 1.2×10^4 (1 plate) and 2.4×10^4 (1 plate) pfu/plate for a total of 36,000 pfu screened. The plates were incubated at 37°C until plaques were approximately 1.5 mm in diameter (12 hours). Plaque lifts and washes were performed by the method described in Maniatis (Maniatis et al., 1989). The filters were prepared in pre-hybridization solution (50% formamide, 25% 20X SSC, 5% 100X Denhardt's solution, 5% 1 M phosphate, 5% denatured salmon sperm DNA, and 0.0625% SDS) for 6 hours at 42°C. Hybridization of the multiprimed DNA fragment (PCR product labelled with 50 μCi [α-^{32}P]dCTP) was done in fresh hybridization solution at 42°C for 12 hours. The filters were then washed for 5 minutes (twice) in 1X SSC/0.5% SDS and for 20 minutes in 0.1X SSC/0.1% SDS.

Mini-preparations of plasmid DNA: Mini-preps were performed by the alkaline lysis method as described in Maniatis (Maniatis et al., 1989).

Large scale plasmid preparations: In cases where large amounts of plasmid DNA were needed, 250 ml bacterial cultures yielded approximately 750 µg DNA according to the PEG-8000 DNA preparation protocol in Maniatis (Maniatis et al., 1989).

Restriction endonuclease digestion: Restriction digests were performed in 20 µl volumes with 0.5 - 1.0 µg DNA, 0.1 volume 10X reaction buffer, 1-10 units of restriction enzyme (depending on application), and water to volume. The digests were performed at 37°C for 1 hour and electrophoresed on a 0.7% agarose gel.

Probe labelling: 250 ng DNA was labelled in a 50 µl total volume with 50 µCi [α-^{32}P]dCTP according to the instructions provided with the kit.

Southern analysis: Southern blotting and probing were performed as described by Maniatis (Maniatis et al., 1989).

DNA sequencing: Double stranded DNA sequencing was performed as follows. 3 µg DNA was dissolved in 5 µl ddH_2O. To this was added 1 µl 100 ng/µl sequencing primer and 1 µl 0.5 M NaOH. The reaction was heated to 70°C in a water bath and allowed to cool

to 25°C over a period of no less than 30 minutes. To this, 1 µl 0.5 M HCl was added, making sure that the acid precipitated DNA was resuspended. From here, the protocol provided with the U.S.B. sequenase kit was followed as though the DNA had just been resuspended after annealing. Single stranded DNA sequencing was performed using the protocol in Maniatis (Maniatis et al., 1989).

Linker kinasing: 2 µg linker was kinased in a total volume of 25 µl using 2 units T4 polynucleotide kinase in 60 mM Tris HCl, 1 mM ATP, 1 mM spermidine, 10 mM $MgCl_2$, 15 mM DTT. The reaction was incubated at 37°C for 45 minutes.

Freeze-squeeze DNA purification: A gel slice containing the DNA fragment of interest was excised from a GTG agarose gel and minced into tiny fragments. 200 µl TE and 400 µl TE-saturated phenol was added with the minced gel fragments to an eppendorf tube. This mixture was frozen on dry ice/EtOH for 5-10 minutes, then warmed to 37°C. The sample was then vortexed for 1 minute, spun in a microfuge for 5 minutes, and the aqueous phase removed. One extraction in phenol/chloroform and one extraction in chloroform was performed. 0.5 volume NH_4OAc and 2 volumes EtOH were added, and the DNA precipitate collected by centrifugation.

Phosphatase treatment: 10 µg of restriction enzyme cut DNA was treated with 10 units of calf intestine alkaline phosphatase (CIAP) in 1 mM $ZnCl_2$, 1 mM $MgCl_2$, 10 mM Tris HCl pH 8.3 for 30 minutes at 37°C. Another 10 units were added and the reaction was incubated another 30 minutes at 37°C before extraction and precipitation of DNA.

Ligation reactions: Blunt-end ligations were performed on 1 µg CIAP treated DNA in 66 mM Tris HCl pH 7.6, 5 mM $MgCl_2$, 5 mM DTT, 100 mg/ml BSA, 1 mM $(NH_3)_6Co^+Cl^-$, 0.2 mM ATP, 0.5 mM spermidine with 2 units T4 DNA ligase for 2 hours at room temperature. Overhanging-end ligations were performed on 1 µg DNA in 50 mM Tris HCl pH 7.6, 10 mM $MgCl_2$, 10 mM DTT, 50 µg/ml BSA. The reaction was first heated to 37°C for 5 minutes to denature pre-ligation sticky-end annealing. ATP was added to 10 mM and 2 units T4 DNA ligase were added. The reaction was incubated at 16°C for 16 hours.

Construction of $SRP54^{Sp}$: The 5.8 kb EcoRI fragment from the λ clone, carrying the $SRP54^{Sp}$ gene (Figure 9) and substantial amounts of both 5' and 3' flanking sequences, was ligated into the EcoRI site of the polylinker of pTZ18U (a kind gift of Byron Kemper).

Construction of SRP54^{Sp}DEL: The 1.1 kb NheI fragment (Figures 9,10) was excised and the complementary ends ligated together with T4 DNA ligase.

Construction of SRP54^{Sp}LEU: SRP54^{Sp}DEL was opened up with NheI and treated with Klenow in the presence of 2.5 mM dNTP's to fill in the overhanging ends. XhoI linkers were kinased and annealed. Vector and linkers were ligated according to the blunt end ligation protocol. This reaction was digested with XhoI to remove excess linkers. DNA was extracted and precipitated. The DNA was then treated with T4 DNA ligase and transformed into bacterial strain NM-522 according to the $CaCl_2$ method and plated out onto 2xYT plates (1.6% bactotryptone, 1.5% agar, 1% yeast extract, 0.5% NaCl, NaOH to pH 7.5) and incubated at 37°C for 12 hours. Isolated bacterial cultures were mini-prepped, digested with XhoI, and analyzed on an agarose gel, confirmed the linkering. Plasmid YEp13 (Figure 10) was digested to completion with PstI, XhoI, and SalI. That digestion along with the linkered SRP54^{Sp}DEL construct were ligated together. This ligation was transformed, mini-prepped, digested, and analyzed on a gel to find the proper construct (Figures 9,10).

Construction of pUTZ4: Plasmids pIRT2 (a kind gift of David Beach) and pURA4 (a kind gift of Paul Russel and Paul Nurse) (Figure 11) were separately digested with HindIII to completion. The 1.76 kb fragment of pURA4 and the backbone of pIRT2 were separately isolated on preparative mini-gels and purified by the freeze-squeeze method. The 2 DNA fragments were then ligated together, transformed, mini-prepped, digested, and analyzed on a mini-gel to find the proper construct.

Construction of pUTZ-54K+: The plasmid pUTZ4 (Figure 11) was digested with BamHI to completion. The plasmid $SRP54^{Sp}$ was digested with BglII to completion. The 4.2 kb fragment from $SRP54^{Sp}$ was isolated by the freeze-squeeze method and ligated to the complementary BamHI ends of pUTZ4. This ligation was transformed, mini-prepped, digested, and analyzed on an agarose gel to isolate the proper construct.

LiOAc transformation of yeast: The S. pombe diploid strain SP629 containing the $SRP54^{Sp}$ knockout (ade6-210/ade6-216 leu1-32/leu1-32 $ura4^-/ura4^-$ $SRP54^{Sp-}/SRP54^{Sp+}$) was transformed with $SRP54^{Sp}$ constructs in the following manner. Cells were grown to 1.0 x 10^7 cells/ml in EMM^2 + uracil liquid media (Mitchison, 1970). Cells were spun down and washed in 0.2 volumes LTE (0.1 M LiOAc, 10

mM Tris HCl pH 7.4, 1 mM EDTA). Cells were spun down, resuspended in LTE at 5 x 10^8 cells/ml and incubated at 30°C for 30 minutes. Addition of 2 µg DNA and 350 µl PEG solution (50% PEG-3500, 10 mM Tris HCl pH 7.4, 1 mM EDTA) was followed by incubation at 30°C for 30 minutes and heat shocked at 42°C for 20 minutes. 0.5 and 0.05 volumes of this mixture were plated onto EMM[2] media with no supplements. Colonies that grew on these plates were diploids that contain the knockout (Leu^+) and the plasmid containing the mutant (Ura^+).

Gene disruption: In collaboration with Steve Althoff, an MD/Ph.D. student in our lab, the $SRP54^{Sp}LEU$ construct—which both removed an entire domain of the protein and conferred leucine prototrophy to the SP629 S. pombe strain—was used to perform the disruption. $SRP54^{Sp}LEU$ was digested with BglII. The 5.2 kb fragment containing the gene knockout construction with 300 nt of 5' flanking sequence and 1.8 kb of 3' flanking sequence was purified by the hydroxyapatite/gel filtration method of DNA purification (Maniatis et al., 1989). This purified fragment was then transformed into SP629 by the spheroplasting method (Beach et al., 1982). Random spore and Southern analysis were used to confirm

integration of SRP54^{Sp}DEL into the SRP54^{Sp} locus. Southern blots were probed with a random primed copy of SRP54^{Sp}DEL.

Random spore analysis: Transformants were placed into 1 ml of 100 mM NaOAc (pH 5.2) with 20 µl glusulase added to remove the ascus walls. After 24 hours of incubation at 30°C, the mixture was plated onto YEA plates (Gutz et al., 1974) and allowed to grow at 30°C for 4-5 days. The individual colonies were streaked onto another YEA plate, grown for one day, and replica plated onto selective media.

Site directed mutagenesis: 3 ng kinased oligo was annealed to 1.5 µg single stranded pUTZ54K+ DNA at 70°C in 20 µl total volume containing 1X sequenase buffer. This was allowed to cool to room temperature in a water bath. The elongation reaction was performed in a total volume of 40 µl containing 5 mM DTT, 1 mM (each) dNTP's, 1 mM ATP, 2 units sequenase, and 2 units T4 DNA ligase. This mixture was set on ice for 5 minutes, set at room temperature for 5 minutes, then incubated at 37°C for 6 hours. The mutagenesis reactions were then transformed into bacterial strain BMH71-18mutS and plated onto 2xYT plates containing ampicillin. Mutants were assayed by double stranded sequencing using the protocol supplied by the manufacturer (U.S. Biochemicals).

RESULTS AND DISCUSSION

Strategy for Cloning $SRP54^{Sp}$: Peter Walter's lab at U.C.S.F. cloned the $SRP54^{Sp}$ gene as a 3 kb SstI fragment with very little flanking sequence, especially on the 3' end. In order to create a genetic disruption of the gene in S. pombe, we needed to clone a larger fragment; about 1 kb on either side of the selectable marker is generally desirable in this organism (Russell, 1990). Oligonucleotide primers M1 and M2 were originally designed by Pat Brennwald, a former graduate student in our lab, according to the published sequence of $SRP54^{Sp}$ (Hann et al., 1989). A 690 base pair sequence of $SRP54^{Sp}$ was amplified by the polymerase chain reaction (PCR) and inserted behind a trp promoter and a fragment of the trpE gene to allow the over-expression of the M domain of $SRP54^{Sp}$ (Brennwald et al., 1991). I used M1 and M2 as PCR primers to test a number of S. pombe DNA libraries for the presence of $SRP54^{Sp}$. All libraries gave a band of the proper size (Figure 4 A).

Isolation of the λ clone: The λ-DASH library was chosen as the vector from which to do the subcloning due to the rapid and easy screening procedure. The λ plating was successful; it showed the proper number of hybridizations for the size of the insert relative to

the size of the S. pombe genome. To isolate the SRP54^{Sp} gene, approximately 36,000 plaques were screened. Assuming an average size of 16 kb, this represents approximately 36 genome equivalents. The probe hybridized to 39 plaques (Figure 5), suggesting that SRP54^{Sp} is present as a single-copy gene. The DNA fragment used to probe the filters containing the λ DNA was the 690 base pair fragment generated by PCR as described above, which was labeled by random priming in the presence of [α-^{32}P]dCTP. Eight plaques were isolated and tested by PCR for the presence of the SRP54^{Sp} insert (Figure 4 B). Seven did contain SRP54^{Sp}. Numbers 3, 4, 5, and 6 were chosen to proceed with further purifications. Three subsequent plaque purifications were done to ensure that the only λ insert I was subcloning was SRP54^{Sp}. Figure 5 shows the last plaque purification which shows no convincing positives for 54-5; therefore subcloning was continued with the 54-3, 54-4 and 54-6.

Large λ DNA preparations were made to isolate material for the subsequent DNA subclonings. This DNA was digested with EcoRI, BamHI, SstI, and PstI and Southern blot analysis performed (Figure 6). Each λ clone contained the exact same Southern profile. The EcoRI digest contains a doublet due to incomplete digestion. Since we wished to have extensive 5' and 3' flanking sequences, and since the

SstI fragment is not much larger than the gene itself, I chose to subclone the EcoRI fragment.

Subcloning of $SRP54^{Sp}$: I took a complete EcoRI digestion of 54-3 and 54-6 and ligated it into the EcoRI site in the polylinker of pTZ18U. To test for the presence of the $SRP54^{Sp}$ insert, I performed colony hybridizations on 398 colonies each of 54-3 and 54-6. The autoradiographs showed several positives on each plate (Figure 7). Six colonies were chosen from each. These were restriction mapped, and one from each clone was sequenced for positive identification. The clone 54-3.5 (now $SRP54^{Sp}$) was chosen for further analysis. A battery of restriction enzymes were employed to make a detailed restriction map. These digests were then Southern blotted to aid in locating the coding sequence (Figure 8).

Knockout strategy: In order to make a construct capable of genetically disrupting the function of this protein, we wished to eliminate as much of the coding sequence as possible while still maintaining the 5' and 3' flanking sequences. In order to do this, the restriction enzyme NheI was used to remove a 1.1 kb fragment of the gene and 187 base pairs of 3' flanking sequence (Figures 9,10). The resulting plasmid was named $SRP54^{Sp}$DEL. To test for the disruption in vivo, either a drug resistance or nutritional marker

must be employed. The Saccharomyces cerevisiae LEU2 gene was chosen. This gene complements the S. pombe leu1 mutation found in the strain SP629. LEU2 was isolated from the plasmid YEp13 (Figure 10) as an XhoI/SalI fragment. SRP54^{Sp}DEL was linkered to accept LEU2, and SRP54^{Sp}LEU was born. The BglII fragment of SRP54^{Sp}LEU was used to make the genetic disruption.

Genetic disruption of SRP54^{Sp} (Figure 13): Recombination in S. pombe is not 100% homologous, so extensive screening must be done to make sure that the knockout construction has been inserted into the proper locus. After the linear BglII fragment was purified, it was transformed into S. pombe strain SP629 by the spheroplast transformation method. The transformants were plated onto EMM^2 minimal media supplemented only with uracil. SRP54^{Sp}LEU colonies that grew were diploids which contained some form of the BglII fragment, since SP629 cannot grow on minimal media that has not been supplemented with leucine. The LEU2 gene can have one of three fates at this point in time. First, it can be maintained as an extrachromosomal element; second, it can recombine at the proper locus; or third, it can recombine at a non-homologous position. The first case is eliminated by curing the yeast of the extrachromosomal LEU2 gene by growth for several generations in media containing 2X

leucine. Removal of selection for the LEU2 gene causes the cells to eliminate it. At this time, the cultures were plated onto rich media. Diploids were picked to minimal plates supplemented with uracil. Only in stable transformants do one hundred percent of the cells form colonies. As an assay to show that the disruption took place, random spore analysis was performed. Table I illustrates that the disruption has occurred, and that $SRP54^{Sp}$ in essential. Upon sporulation, the Leu+ knockout strain heterozygous for the disruption gave rise to no Leu+ haploids since the LEU2 gene segregates with the disrupted copy of $SRP54^{Sp}$. When transformed into the knockout strain, the wild type $SRP54^{Sp}$ contained on the plasmid pUTZ54K+ complements the disruption, suggesting that lethality was due solely to the disruption of $SRP54^{Sp}$. To ensure that proper recombination occurred, Southern blot analysis was performed (Figure 14). The wild type BglII fragment appears as a 4.2 kb band, and the knockout construct as a 5.3 kb band, confirming that the diploid contains one copy of each. In combination, these experiments provided definitive proof that the disruption was successful and that $SRP54^{Sp}$ is essential for viability of S. pombe. We previously showed that the 7SL RNA component of SRP is essential in S. pombe (Brennwald et al., 1988). The budding yeast Saccharomyces cerevisiae SRP54 homologue

($SRP54^{Sc}$), as well as the RNA component of S. cerevisiae SRP, are not essential.

Mutational analysis of the GTP domain of $SRP54^{Sp}$: The highly conserved amino acid sequences of GTPase proteins (reviewed in Bourne et al., 1991; Figure 3) suggests that a strict structural and spatial arrangement of the GTP binding domain must be maintained for GTPase function. There are four discrete regions, designated G-1 through G-4, which participate in the GTPase function of a G protein (Figure 3). The roles of each of these have been ascertained from the X-ray crystal structure of $p21^{ras}$ (Bourne et al., 1991). The G-1 region is responsible for the binding of the α- and β- phosphates of GTP. The G-2 region is responsible for coordinating a Mg^{2+} ion within a pocket that also contains the β- and γ- phosphates of GTP. This Mg^{2+} ion is absolutely essential for GTP hydrolysis. G-3 contains an invariant Asp residue that is involved in coordinating the Mg^{2+} ion through an intervening water molecule. It also contains an invariant Gly that is hydrogen bonded to the γ phosphate of the GTP. G-4 has been shown to hydrogen bond to the guanine ring, stabilizing the GTP during catalysis.

As noted in the Introduction, SRP54 contains a GTP binding motif. $SRP54^{mam}$, in conjunction with the mammalian SRP receptor

and SRP RNA, has been shown to hydrolyze GTP (Poritz et al., 1990). The G-4 domain of the SRP54 GTPases, along with dynamin and VPS1 (Obar et al., 1990), have one striking difference from other G proteins: specifically, the single letter amino acid code for the G-4 consensus is TKXD in these GTPases proteins, rather than NKXD as in the $p21^{ras}$ consensus (Figure 3). Since there is a divergence at this residue, I decided to mutate it to both a conservative and a non-conservative amino acid to determine the effects on $SRP54^{Sp}$ function. The threonine at amino acid position 248 (T248 in $SRP54^{Sp}$) was changed by site directed mutagenesis, conservatively to a serine (T248S), and non-conservatively to an arginine (T248R) (Table I). The strategy for assaying mutants in the knockout strain is illustrated in Figure 15. As shown in Table I, the mutant T248S complements the disruption, thus conferring viability to the spores which contain the disrupted copy of $SRP54^{Sp}$. This suggests that a small, conservative change in that amino acid does not interfere with the function of the protein. T248S was assayed for temperature sensitivity, cold sensitivity, and osmotic temperature sensitivity (Liao et al., 1989) and had no conditional phenotype. In contrast, the mutant T248R was not viable. As arginine is not at all a conservative change, one might suggest that the asparagine or threonine residue

present in wild type GTPases, as well as the serine in T248S mutant, are able to hydrogen bond to the guanosine ring, while the bulky, charged arginine prevents nucleotide entry and/or binding to that region of the GTP binding domain. It will be especially interesting to see whether changing the threonine to asparagine, as found at the corresponding position of $p21^{ras}$ and EF-Tu, gives a functional $SRP54^{Sp}$ protein. I am currently creating this mutation.

In summary, I have found S. pombe to be a excellent organism with which to do my experiments. The ability to genetically manipulate this organism makes studying protein secretion very feasible. Most proteins studied in higher eukaryotic in vitro systems depend on SRP for translocation. S. pombe SRP closely resembles mammalian SRP structurally. Thus, since SRP components are essential in S. pombe, but not in S. cerevisiae, results derived from this particle in S. pombe should provide insights into higher eukaryotic SRP function.

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Spore	YEA	Ura+	Leu+/Ura+	Leu+/Ura-
Knockout	66	0	0	0
Wild type SRP54	66	61	37	0
SRP54 T248S	66	63	38	0
SRP54 T248R	66	59	0	0

Table 1.. Random spore data for complementation with wild type $SRP54^{Sp}$ and mutant derivatives.

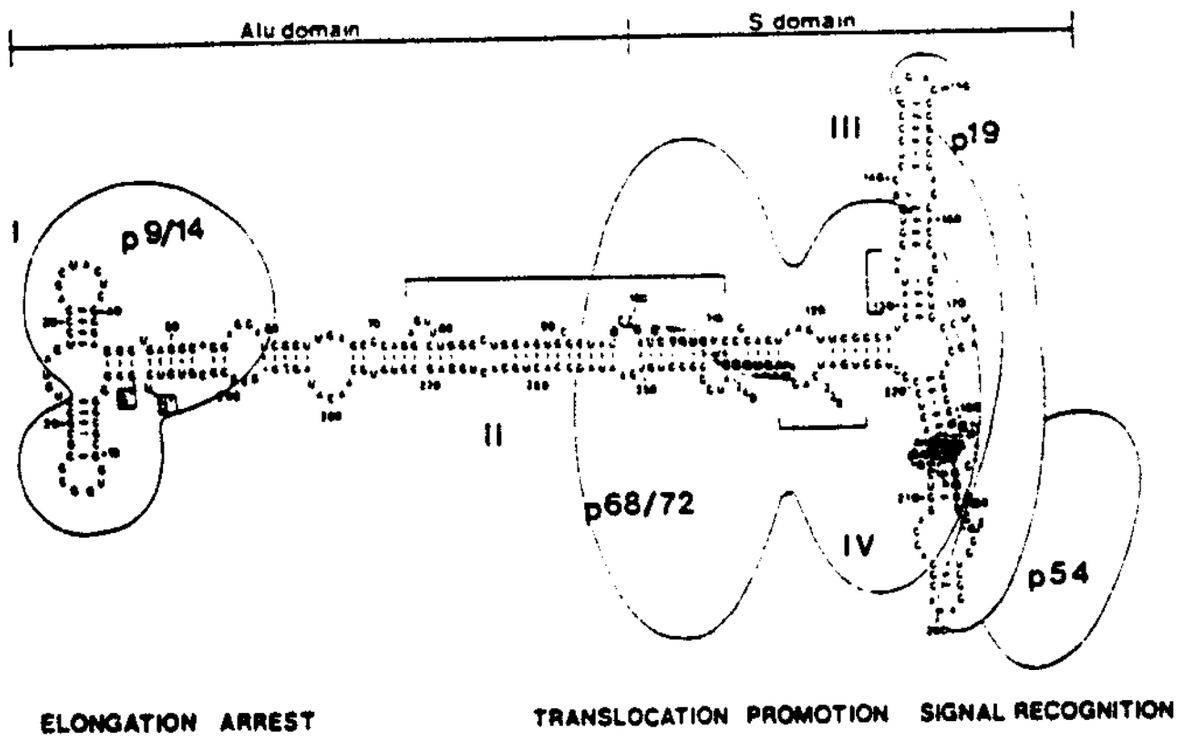


Figure 1. Secondary structure of mammalian 7SL RNA and position of SRP proteins (taken from Andreazzoli and Gerbi, 1991).

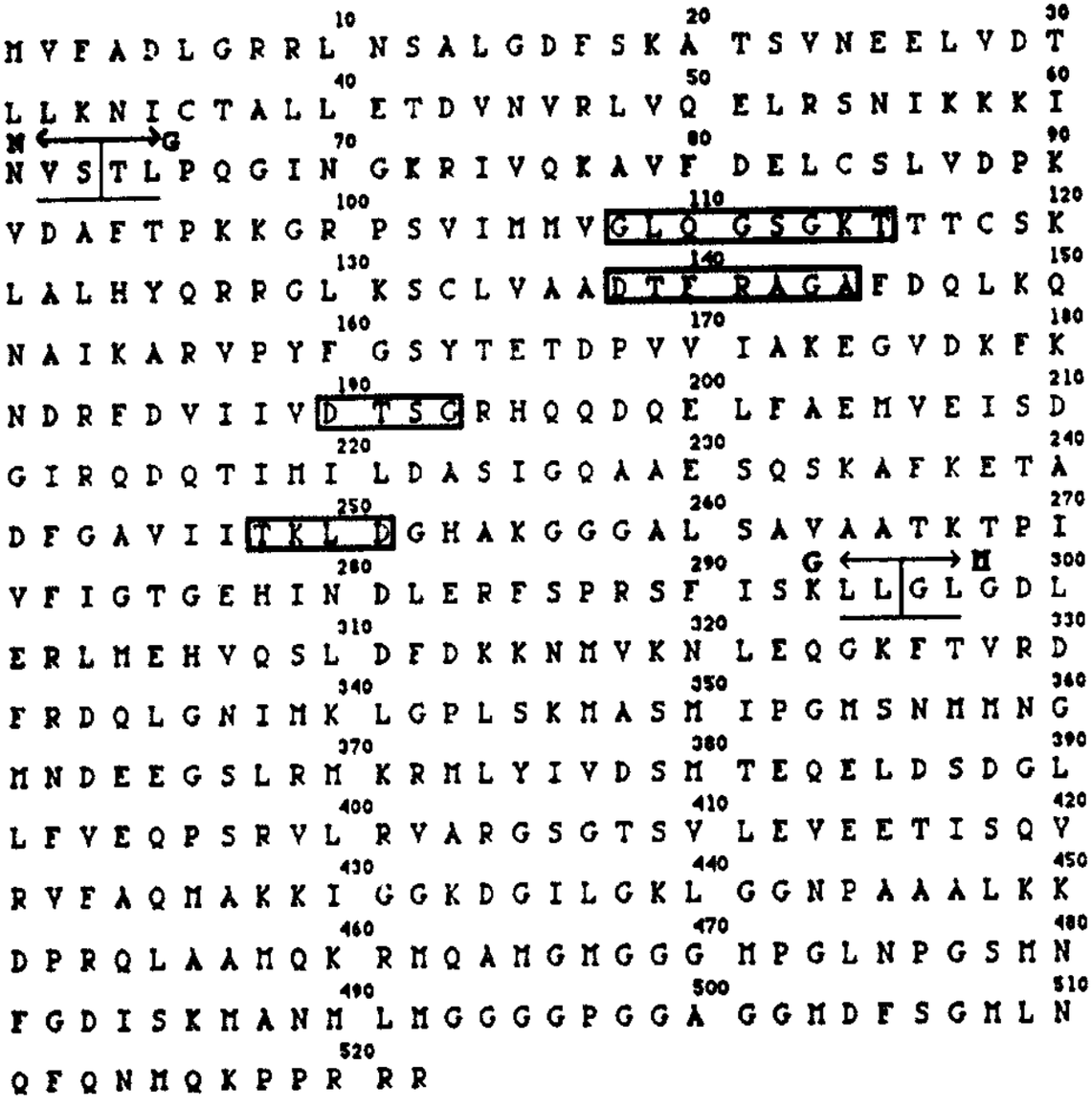
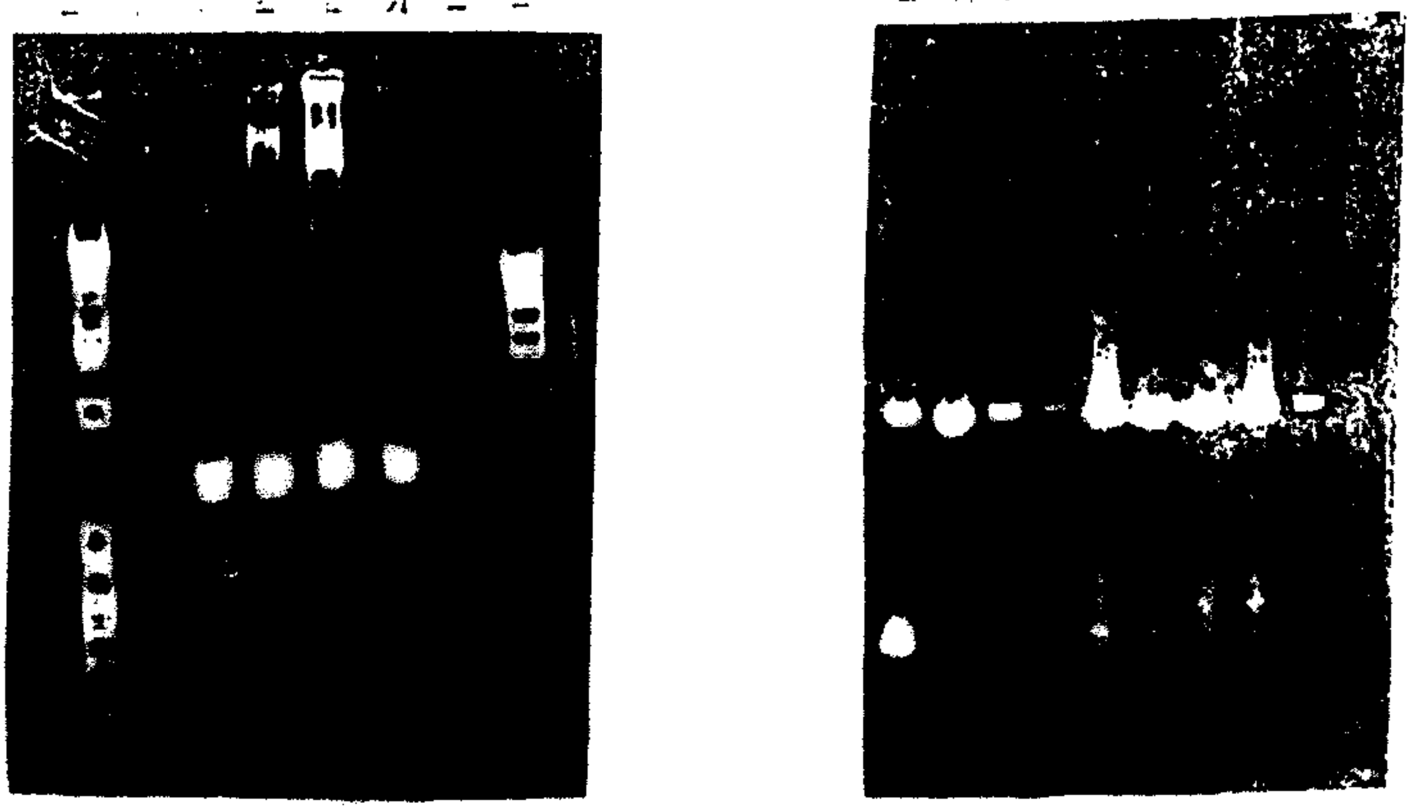


Figure 2. Predicted amino acid sequence of S. pombe SRP54^{Sp}. GTP binding domains are boxed. N, G, and M domains are delineated.

	G-1	G-2	G-3	G-4
GTPase consensus	XOOOOGXXGXGKS	D-(X)n-T	OJOODXAGJX	OOOOTKXD
SRP54mam	vimivGLQGSGKT	DTFRAGA	iiivDTSGrh	avivTKLD
SRP54sp	iimfvGLQGSGKT	DTFRAGA	iiivDTSGrh	aiilTKLD
SRP54sc	vimmvGLQGSGKT	DTFRAGA	viivDTSGrh	aviiTKLD
E.coli ffh	vvlmaGLQGaGKT		vllvDTaGrl	gvvlTKvD

X - Any Amino acid residue
O - Hydrophillic residue
J - Hydrophobic residue

Figure 3. **Boxes G-1 through G-4 of GTP binding domains of the GTPase consensus sequence (Bourne et al., 1991), $SRP54^{mam}$, $SRP54^{Sp}$, $SRP54^{Sc}$, and ffh.**



A.　　　　　　　　B

Figure [illegible] [illegible] PCR
A. PCR reactions [illegible] of SRP57. See Materials and Methods for details.
B. PCR reactions to test [illegible] of SRP57 [illegible] clone.

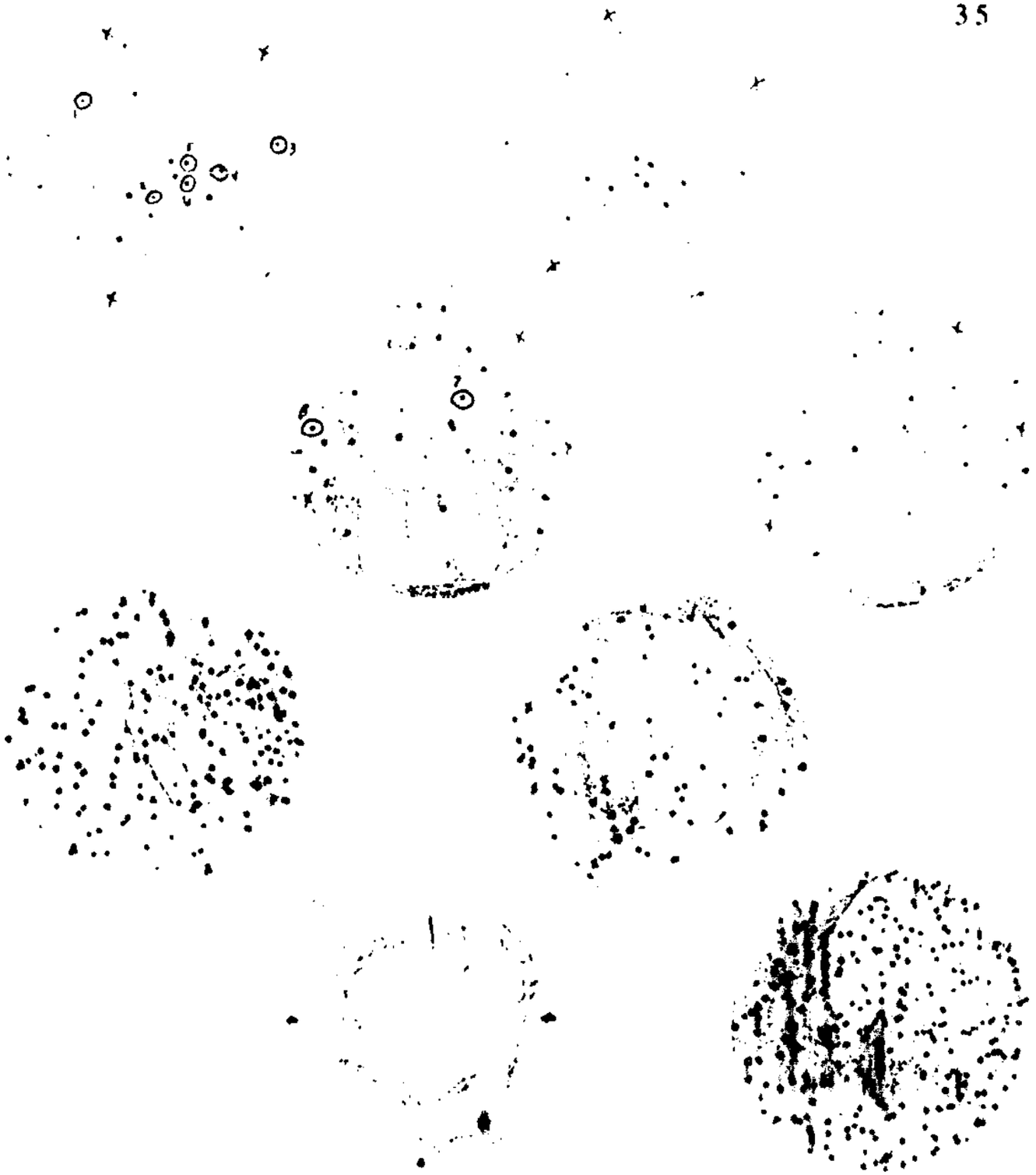
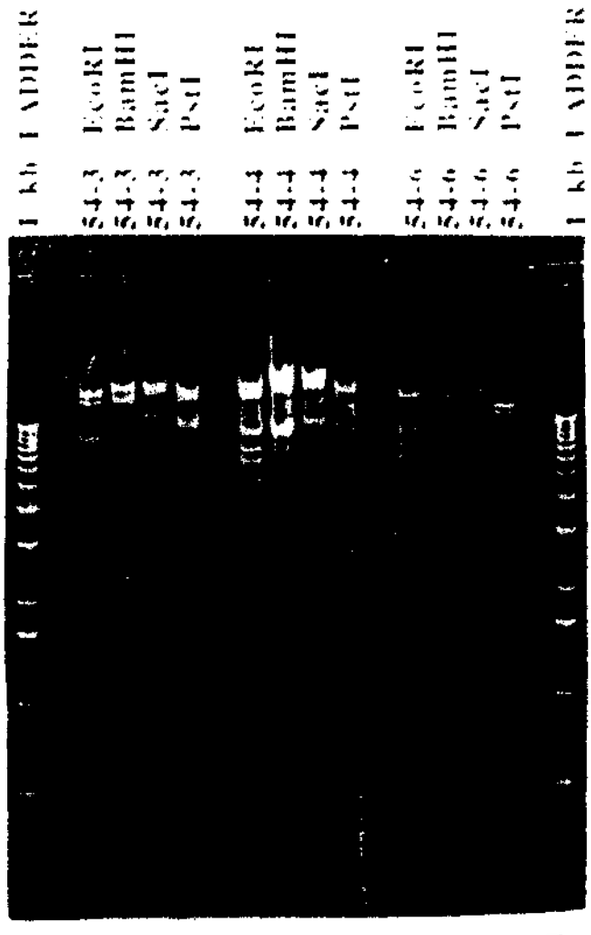


Figure 5. Plaque hybridizations. Top 2 filters are duplicates containing 1.2×10^4 plaques/plate. Next 2 filters are duplicates containing 2.4×10^4 plaques/plate. Circled plaques were picked. Bottom 4 filters are the final plaque purifications. All plaques on the plate gave signals detectable in the autoradiograph.

A.



B.

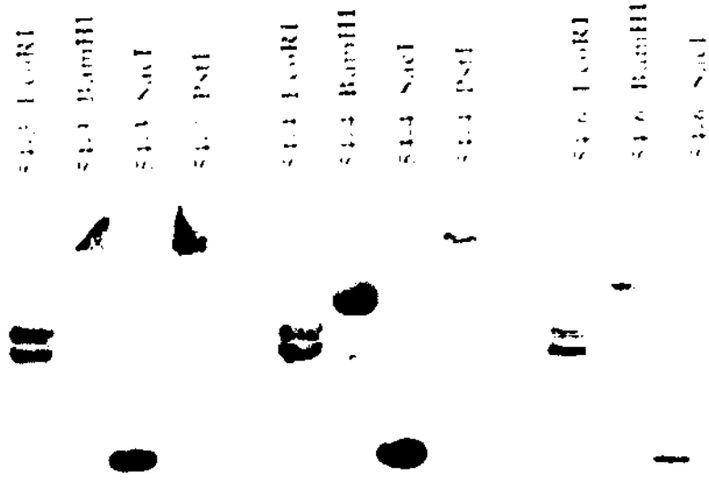


Figure 6. A. Agarose gel of 7 DNA digests. See Materials and Methods for details.

B. Southern blot of A. probed with the random primed PCR fragment. 2 bands light up in the EcoRI lane due to incomplete digestion. A 3 kb SacI fragment is indicative of SRP54^{ts} (Hann et al. 1989).

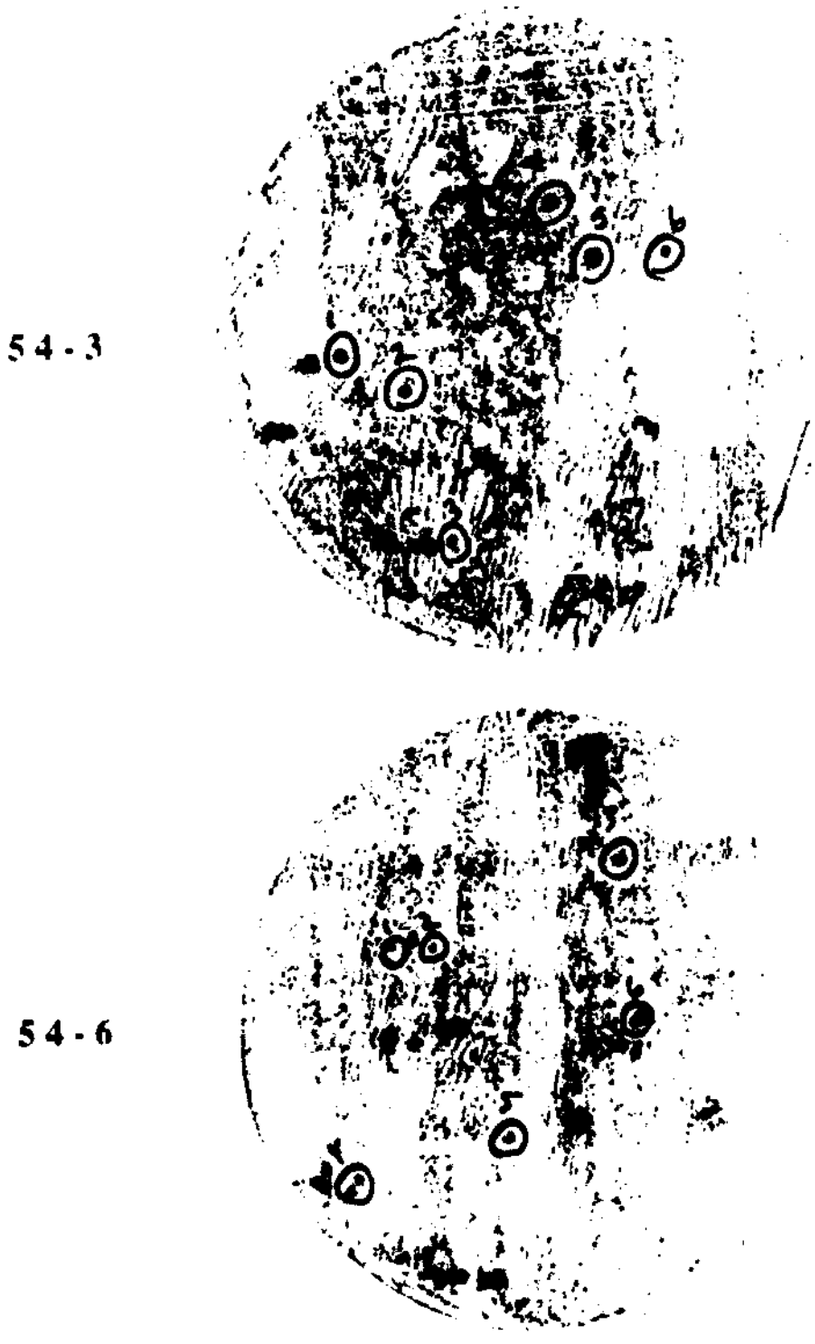
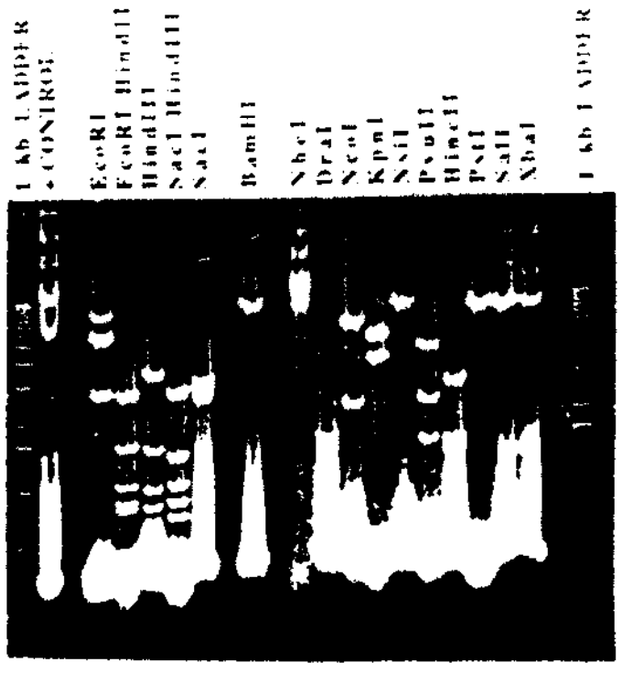


Figure 7. Colony hybridization of $SRP54^{Sp}$ subclone in pTZ18U. DNA was probed with the random primed PCR product.

A.



B.

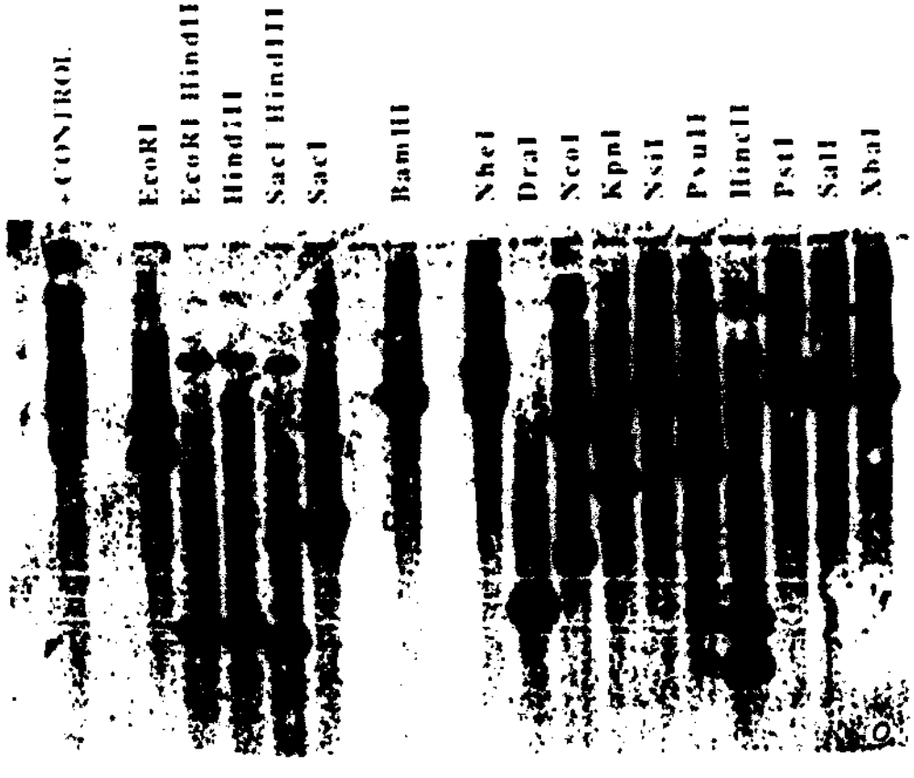


Figure 8. A. Agarose gel of digests of SRP54^{SP} EcoRI clone. See Materials and Methods for details.

B. Southern of A. probed with random primed PCR fragment. Again, the doublet in EcoRI lane is due to incomplete digestion of DNA.

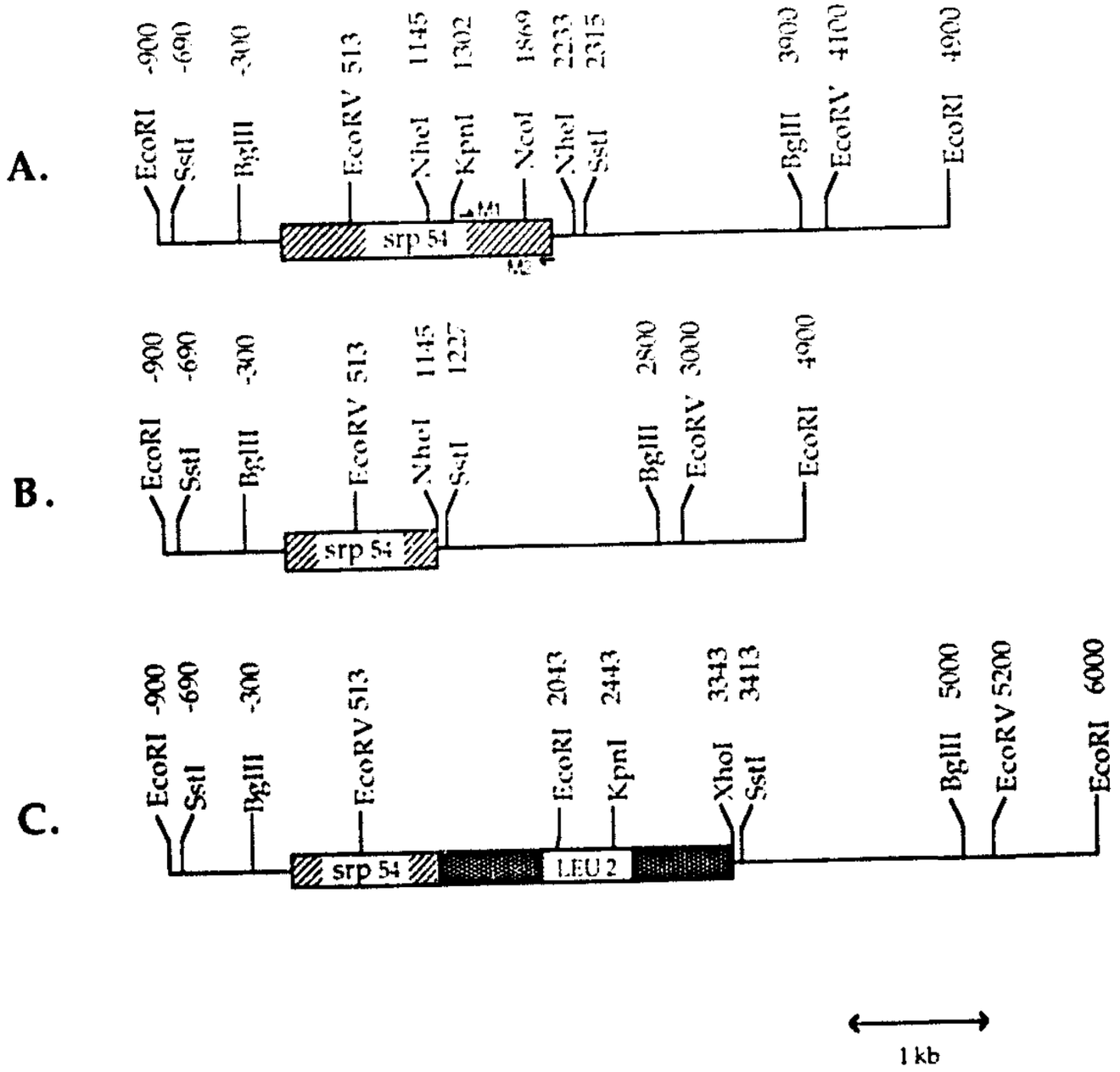


Figure 9. Linear maps of A. SRP54^{Sp}, B. SRP54^{Sp}DEL and C. SRP54^{Sp}LEU. The positions where oligonucleotide primers M1 and M2 hybridize are shown in A.

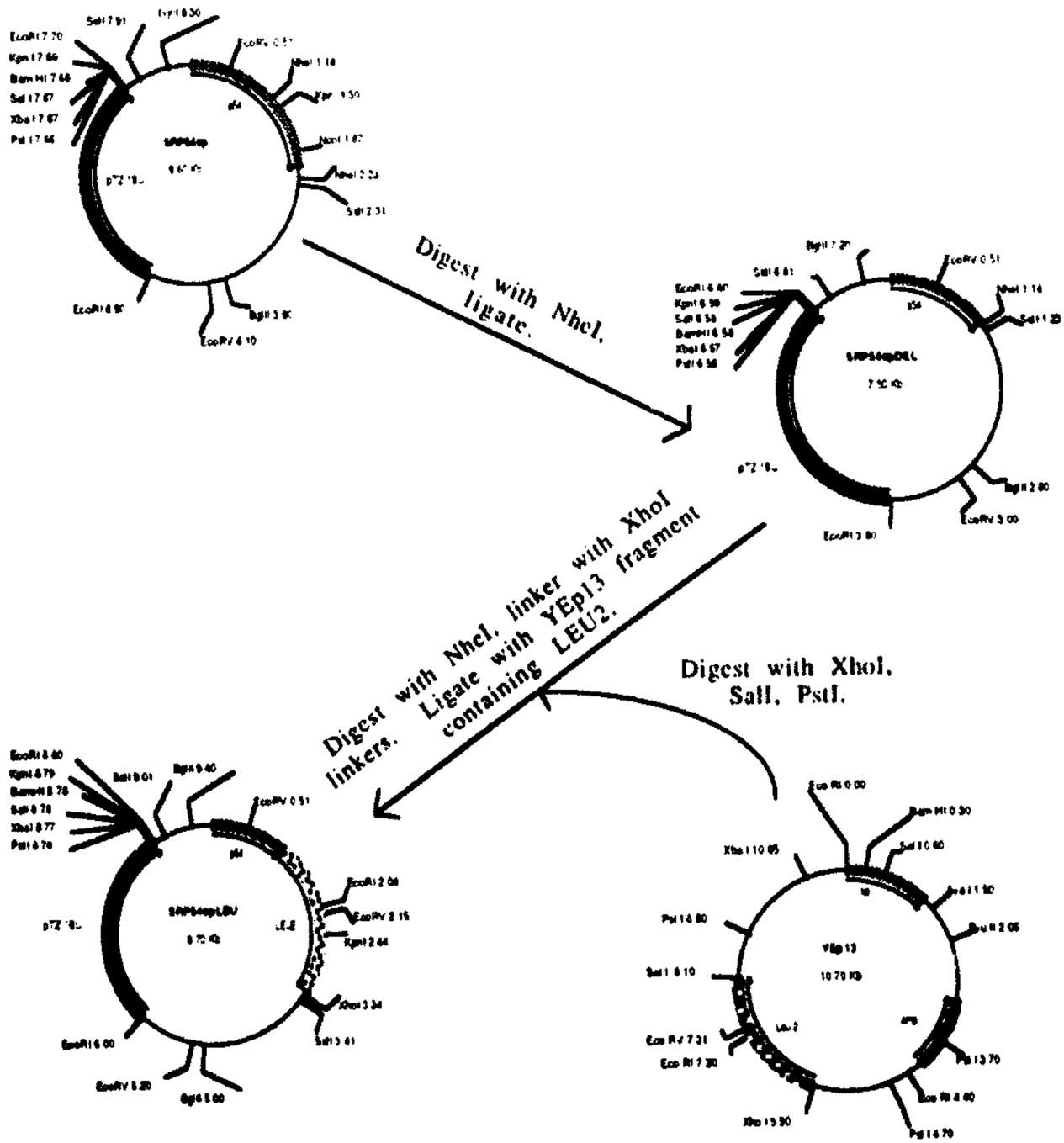


Figure 10. SRP54^{Sp}, SRP54^{Sp}DEL, SRP54^{Sp}LEU, and YEp13.

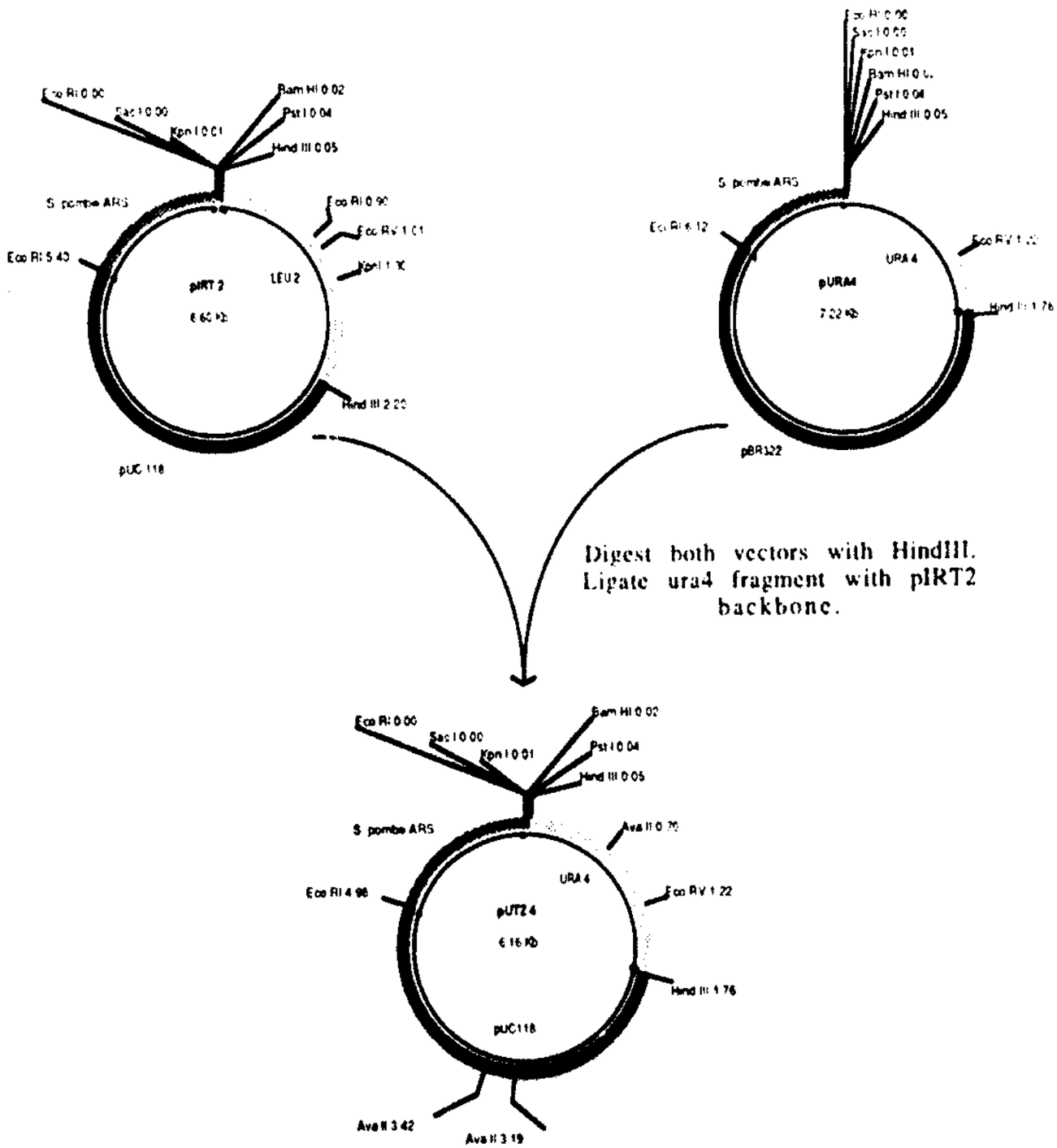


Figure 11. Construction of pUTZ4.

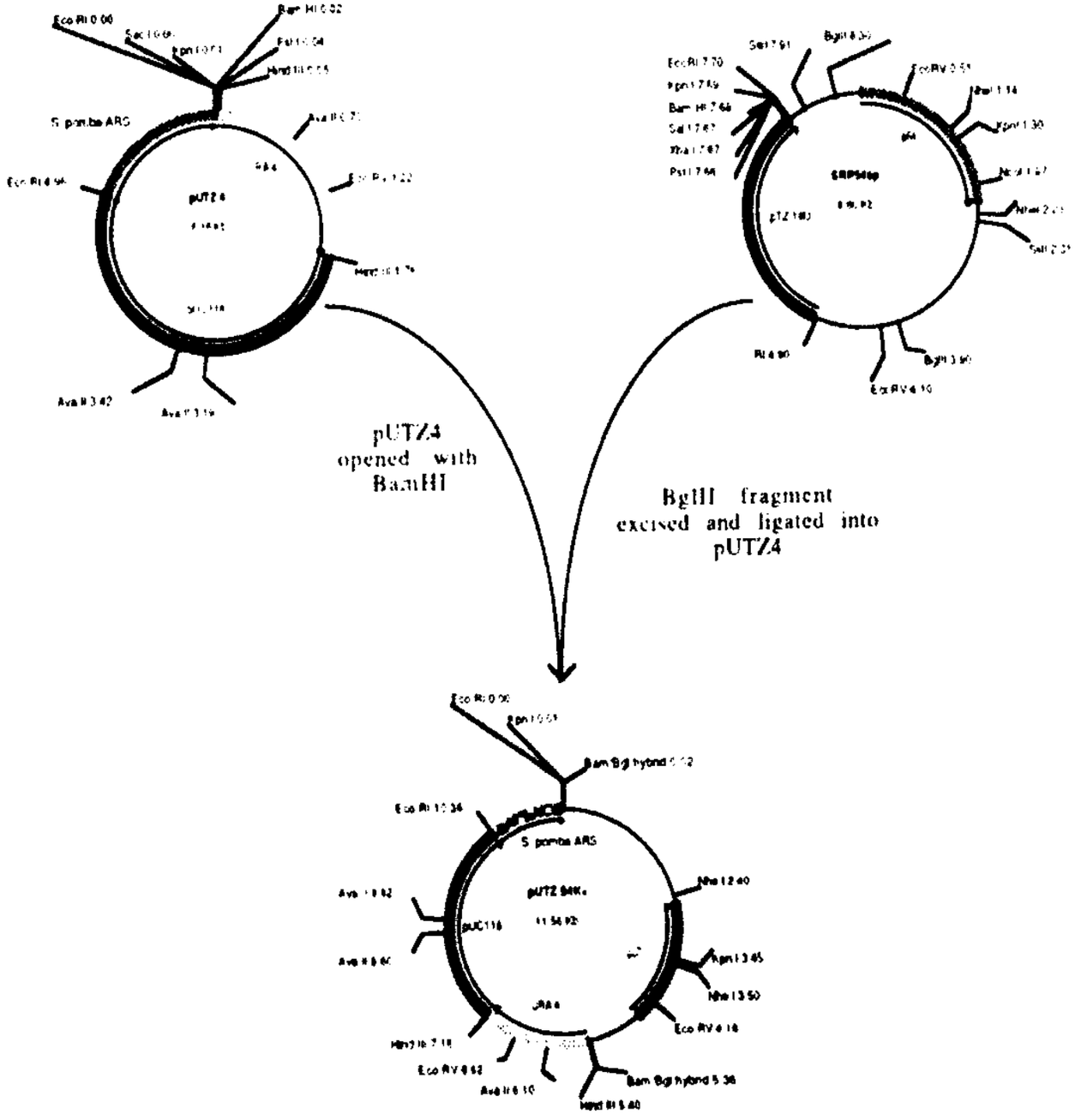


Figure 12. Construction of pUTZ54K+.

KNOCKOUT STRATEGY FOR SRP54

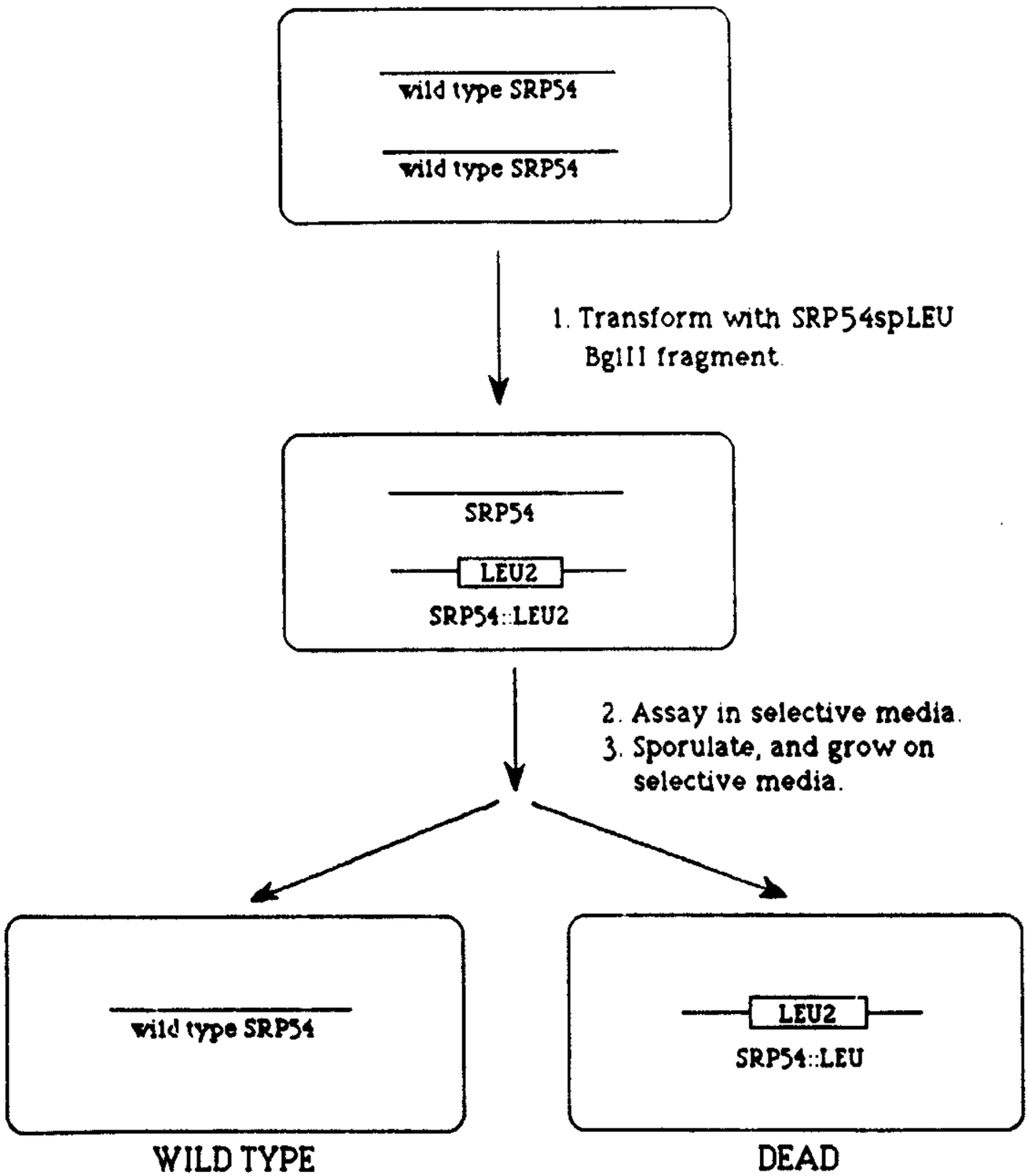


Figure 13. SRP54^{Sp} disruption strategy.

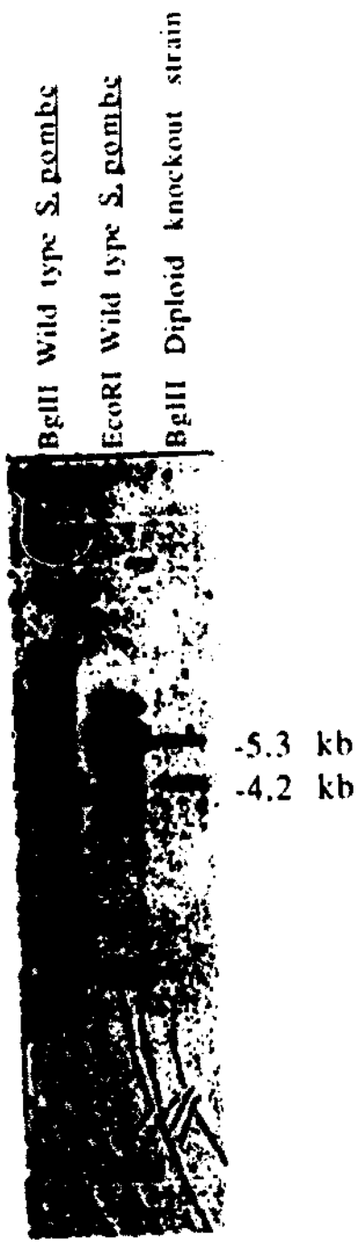


Figure 14. Southern blot of SRP54^{Sp} disruption.

COMPLEMENTATION OF SRP54 DISRUPTION WITH PLASMID BORNE GENES

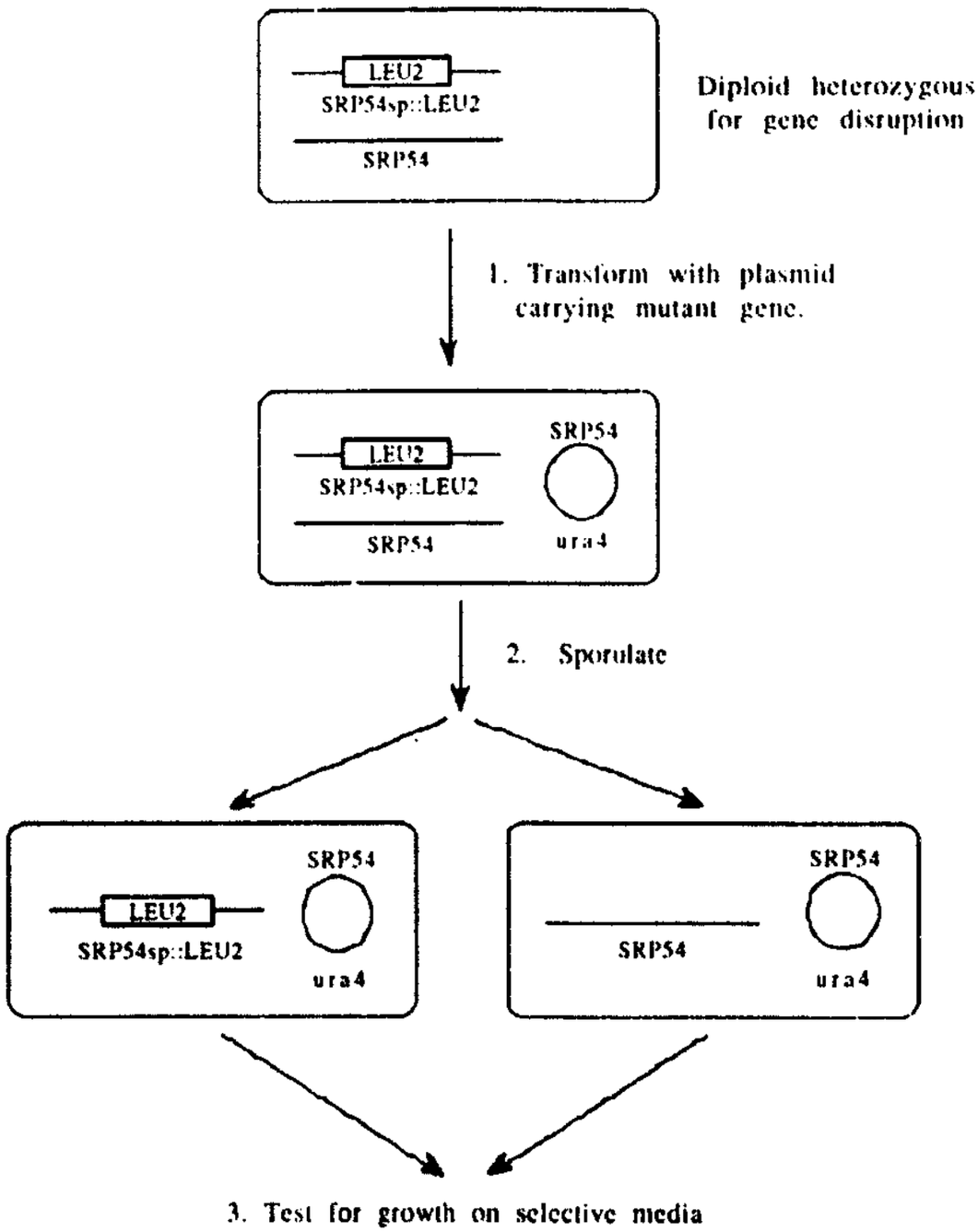


Figure 15. Strategy for complementation of SRP54^{Sp} disruption with the wild type gene, or plasmids carrying point mutations.