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Synthetic lethal/enhancer screening to identify snRNA:protein and protein:protein interactions in yeast pre-mRNA splicing

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1. Introduction

Over the past five years or so, our laboratory has carried out three synthetic lethal screens aimed at identifying novel yeast (*S. cerevisiae*) genes involved in pre-mRNA splicing. Although many of the results have been published (1–5), we have never assembled an overall summary of what we obtained and how well the screens worked to identify new genes—an informal assessment of the genetic signal-to-noise ratio. Otherwise put, what is the benefit-to-labour ratio in taking this approach to the splicing factor identification problem? As our procedures have also undergone modifications as a function of time and experience, this will serve as a useful venue for discussing the advantages and disadvantages of the approaches we have tried.

Synthetic lethality refers to a situation where two mutations (usually in two different genes) are viable when assayed alone but lethal as a double mutant combination. 'Synthetic' means putting two things together. 'Lethality' is an extreme version of the more general situation, where the two mutations together have an unusually strong or weak phenotype compared to one or both mutations assayed on their own. Synthetic lethality can be arranged by the investigator, who combines two mutant alleles in the same strain; if the resulting strain is inviable, the combination is defined as synthetic lethal. Otherwise, the combinations can arise from a genetic screen, where the novel mutant genes are revealed in an already mutant background. The use of a starting strain already deficient in a particular process—the synthetic lethal or suppressor/enhancer paradigm—is designed to increase the target size for genes that contribute to the biochemical process or pathway of interest (6). In general, the strategy is acknowledged to be successful, because it has uncovered genes that would have been difficult to identify in other more straightforward genetic screens.

A single synthetic lethal combination is not very informative about how important each individual gene is for viability. The individual mutations could be in inessential genes with only mild phenotypic effects. For instance, both single mutations could be null mutations with phenotypes indistinguishable from those of gene deletions. As many genes from yeast as well as other organisms are inessential and have little phenotype when deleted (6), this is a realistic possibility. At the other extreme, one or both mutations could be mild loss-of-function alleles in essential genes. In this case, the synthetic lethal screen can provide information that would otherwise require collecting a large number of alleles in the gene.

Synthetic lethality also may not provide an obvious picture of how important the two genes are to the biochemical process or pathway of interest. The lethality can be for uninteresting reasons, i.e. the two mutants can be in completely different biochemical pathways; each alone makes the cells quite sick, and the combination of the two unrelated 'illnesses' leads to death. The two pathways might converge on a single product: product levels would be reduced in one mutant, reduced in the other, and further reduced in the double mutant combination below a threshold required for viability. For example, a mutation in the transcriptional machinery and another mutation in the protein synthetic machinery could have this cumulative negative effect on the level of an important protein. The interesting situation occurs when the two mutants are in the same process or pathway. Indeed, many synthetic lethal screens are designed to identify novel genes contributing to a defined pathway.

Our own work in this area began with the analytical approach, i.e. phenotypic assays of double mutant combinations. We had cloned and analysed the secondary structure of yeast U1 snRNA (7–9). The RNA was much longer than that of mammalian U1 snRNA (865 versus 164 nucleotides) but clearly resembled its mammalian counterpart. Many of the highly conserved sequence elements were apparent (*Figure 1*). These included:

(a) The completely conserved 5′ arm, which undergoes base pairing with the 5′ splice site.

(b) The first stem-loop, which contains the binding site for the U1 70K protein.

(c) The general cruciform structure of the molecule.

(d) The Sm binding site.

Yeast U1 snRNA also contains additional sequence elements which are highly structured, as indicated by computer modelling, structure probing, and most importantly, by phylogenetic comparisons among different yeast species. The molecule therefore contains universal as well as yeast-specific sequence and structural elements (8).

The ease of gene replacement and phenotypic analysis in yeast allows one

Figure 1. Structure of yeast U1 snRNA. The yeast U1 snRNA helices have been numbered from I to X. The U1–4U mutation in the 5′ arm is indicated by an *arrow*. The second U1 snRNA mutant used in the screen (U1ΔYC+LII26A) contains a deletion of the yeast core sequences (ΔYC) and a point mutation in loop A. This double mutant grows slowly.

to ask how a particular molecule contributes to viability and the extent to which it contributes to growth rate (colony size for example). As expected from the phylogenetic conservation (yeast to man) and the importance of the U1 snRNA molecule to *in vitro* splicing, the U1 snRNA gene is essential for viability. However, mutations within the molecule gave rise to more surprising

results. Several mutations within regions conserved between yeast and mammals were without any obvious phenotypic consequence (9). This was puzzling, as their universally conserved sequence features predicted that they would be essential for splicing and therefore for viability. More sensitive reporter gene assays for splicing showed that mutations in these regions had little effect on splicing efficiency. Moreover, some of the yeast-specific regions appeared more important for splicing than some of the universally conserved regions (9).

We considered a number of interpretations, including the possibility that these universally conserved regions might function in some process other than splicing. But we finally realized that the conundrum probably reflected a number of complicated relationships between these different activities *in vivo*: the relationship between the U1 snRNA sequence and U1 snRNP activity, the relationship between U1 snRNP activity and splicing efficiency, and the relationship between splicing efficiency and growth rate. Only when the mutant is catastrophic is the phenotypic consequence predictable: death.

To test these interpretations, we analysed double mutant combinations in U1 snRNA, what we called intramolecular synthetic lethality. The results were as predicted: many of the double mutants had splicing or growth phenotypes much more severe than the individual single mutants. Moreover, double mutant combinations successfully tested structural predictions of the U1 snRNA model (9). The results indicated that one can decrease substantially U1 snRNP activity without any effect on splicing efficiency. They also suggested that one can decrease splicing efficiency substantially without any effect on growth rate. In other words, U1 snRNP activity is normally not rate-limiting for splicing, and splicing is not normally rate-limiting for growth. These conclusions are not surprising in hindsight, as there is no reason to expect that U1 snRNP activity rather than one of the many other steps of splicing be limiting under normal growth conditions nor should a threefold decrease in splicing efficiency necessarily translate into a threefold decrease in growth rate. However, when a first mutation makes U1 snRNP activity limiting for splicing or nearly so, splicing efficiency is then sensitized to respond quasi-linearly to additional even mild decrements in U1 snRNP activity. In some cases, the splicing efficiency is so poor that even growth rate is limiting and responds to further decrements in U1 snRNP activity.

We realized that this logic should equally well apply to intermolecular synthetic lethality and permit the identification of U1 snRNP proteins or other splicing factors that interact functionally with U1 snRNA at these early steps of spliceosome assembly. The strategy was to use some of the same U1 snRNA mutations that were used in the intramolecular combinations and identify mutants that render the starting strain inviable in a U1 allele-specific fashion (1).

Two different approaches have been used in our laboratory to identify new splicing factors that collaborate with components of the splicing machinery.

The first approach is based on a viability assay and was first used to identify mutations which are lethal in combination with mutant variants of U1 snRNA (1). The methods used in this screen are described in Section 2.2. This screen identified MUD1, the yeast homologue of the human U1A protein, as well as MUD2, a component of the splicing commitment complex (1, 2); additional factors identified in this screen are discussed in Section 2.3. This same first approach was then used to identify mutants synthetically lethal with a complete disruption of the non-essential MUD2 protein (10). For reasons discussed in Section 2.3, this latter screen was not very successful. To identify additional components functionally related to MUD2, we took a somewhat different experimental tack based on the red-white sectoring assay (11). The methods and results of this second screen are described in Section 3.

2. U1 snRNA enhancer screen using a viability assay

2.1 General reagents and strains

2.1.1 Plasmids

(a) pGAL-U1: wild-type U1 snRNA coding sequence driven by the GAL1-CYC promoter on a centromeric URA3 plasmid.

(b) pU1mut: mutant U1 snRNA coding sequence driven by its own promoter on a centromeric TRP1 plasmid. The U1 snRNA enhancer screen has been carried out with two different U1 mutants: U1–4U which contains a C to U substitution at the 5' end of U1; U1 ΔYC+LII26A which contains a mutation in U1 A loop as well as a deletion of yeast-specific sequences (*Figure 1*) (1).

2.1.2 Starting strains

(a) XLY 219 : Mat**a**, trp1–289, ura3–52, leu2–3,112, arg4 (RV-), ade2, U1snRNA::ADE2, pGAL-U1, pU1mut.

(b) XLY 216 : Matα , trp1–289, ura3–52, leu2–3,112, his3-D1, cyh^R, U1snRNA::LEU2, pGAL-U1, pU1mut.

2.1.3 Media and plates

All the media and plates are prepared according to standard protocols (ref. 12 and see also Chapter 8). YPD (also referred to as YEPD, e.g. Chapter 8) contains 1% yeast extract, 2% peptone, 2% glucose, and 2% agar for plates. YPG (also referred to as YEPG) plates contain 1% yeast extract, 2% peptone, 1% glycerol (w/v); the pH is adjusted to 5.5 with HCl before adding the 2% agar. YPG selects against spontaneous respiratory mutants which can not use the non-fermentable glycerol. Unless otherwise stated 2% glucose is included in all media and cells are grown at 30 °C.

2.2 Protocols

Protocol 1. Mutagenesis by UV irradiation or EMS

Equipment and reagents

- UV Stratalinker 1800 (Stratagene)
- 50 mM potassium phosphate buffer pH 7, filter sterilized
- Ethylmethanesulfonate (EMS, Sigma)
- 10% sodium thiosulfate solution (w/v), freshly prepared, filter sterilized
- 1 M sodium hydroxide + 0.6% thioglycolic acid

Two different and complementary mutagenesis protocols can be used. UV mutagenesis is simple and not as toxic as EMS; also, it produces both transitions and transversions, in addition to double base changes, unlike EMS which causes only transitions.

A. *Mutagenesis by UV irradiation*

1. Grow the starting strains (*Figure 2A*) overnight in 20 ml of Trp^- Ura^- + 2% glucose medium to an A_{600} of 1–2 (about 2×10^7 cells/ml).
2. Plate roughly 4000 wild-type cells on Trp^- Ura^- + 3% galactose/1% sucrose 100 mm diameter plates. Plate a 10- to 100-fold dilution of the cells on a control plate that will not be irradiated in order to determine the survival rate of mutagenized cells.
3. Place the plates inside the UV Stratalinker without cover lids and with the colonies directly facing the UV light bulbs.
4. Irradiate the cells so as to get 5–15% viability. The extent of irradiation is strain-dependent and should be determined in a pilot experiment; 90 × 100 mjoules of radiation yielded roughly 10% survival for our strain.[a]
5. Cover the plates immediately with their lids and wrap them in aluminium foil to reduce photoreversion of UV-induced changes.
6. Incubate at 30°C for two to three days or until the colonies have a size suitable for replica plating (*Figure 2B*).

B. *Mutagenesis by EMS*[a,b]

1. Grow the starting strain (*Figure 2A*) overnight in 20 ml of Trp^- Ura^- + 2% glucose medium to an A_{600} of 2–3 (about 5×10^7 cells/ml).
2. Pellet the cells (2 × 10 ml) by centrifugation at 2400 *g* for 5 min in 50 ml sterile screw-cap tubes. Wash the pellets twice by resuspending them in 10 ml of 50 mM potassium phosphate buffer pH 7.0, and then recentrifuging them. One sample is a no EMS control.
3. Resuspend in 10 ml 50 mM potassium phosphate buffer pH 7.0.

4. Add 300 µl EMS[a,b] to one 10 ml tube, vortex, and incubate both the EMS and the control tubes for 30 min at 30°C with agitation.
5. Stop EMS mutagenesis by the addition of 10 ml of 10% sodium thiosulfate solution. Mix, and collect the cells by centrifugation as in step 2; wash the pellets twice with sterile water.
6. Plate an appropriate dilution of the mutagenized and control cells on Trp^- Ura^- + 3% galactose/1% sucrose plates, and incubate at 30°C for up to three days, or until the colonies have a size suitable for replica plating (*Figure 2B*).

[a] The survival rate during mutagenesis can be a critical factor. EMS is light-sensitive and its mutagenic activity decreases with time. The EMS dose proposed kills 50–90% of the cells of most strains, but it is usually desirable to check the survival level of the particular strain used. The 10% survival rate produced in our first two screens was probably too high producing strains with multiple mutations (see Section 2.3).
[b] EMS is highly toxic and all the manipulations have to be carried out under the hood. Tips and supernatants shoud be deactivated in 1 M NaOH + 0.6% thioglycolic acid for 24 h and thrown away as toxic waste.

Protocol 2. Identification of synthetic lethal mutants

1. Replica plate the viable UV or EMS mutagenized cells to 5-FOA + 2% glucose plates by transferring the cells on the master plate to a dark coloured sterile velvet wrapped around a cylindrical block, and then transferring the cells from the velvet to the second plate. Incubate at 30°C for one to two days.[a]
2. Compare the master and the replica plates. The missing colonies on the replica plates are identified as having mutations that synergize with the original mutant to cause a lethal phenotype.[a] In our U1 snRNA synthetic lethal screen these were called 'mud' mutants (mutant U1 die) (*Figure 2C*).
3. Pick the corresponding mutant colonies from the master plates with sterile toothpicks and grow them on Trp^- Ura^- + 3% galactose/1% sucrose plates as little patches to check their phenotypes and genotypes.
4. Replica plate the patches to individual drop-out plates containing 3% galactose/1% sucrose, and to 5-FOA, YPD, and YPG plates.
5. Collect the patches that have the same auxotrophic markers as the starting strain and that do not grow on 5-FOA or YPD; freeze as the original mutant stock. Maintain these mutants in Trp^- Ura^- + 3% galactose/1% sucrose medium.

[a] The yeast cells containing a mutation that is synthetic lethal with the U1 snRNA mutation carried by the TRP1 plasmid will not be able to grow on 5-FOA (*Figure 2B*); these cells require the wild-type U1 gene carried by the URA3 plasmid for viability and therefore cannot grow on 5-FOA (23).

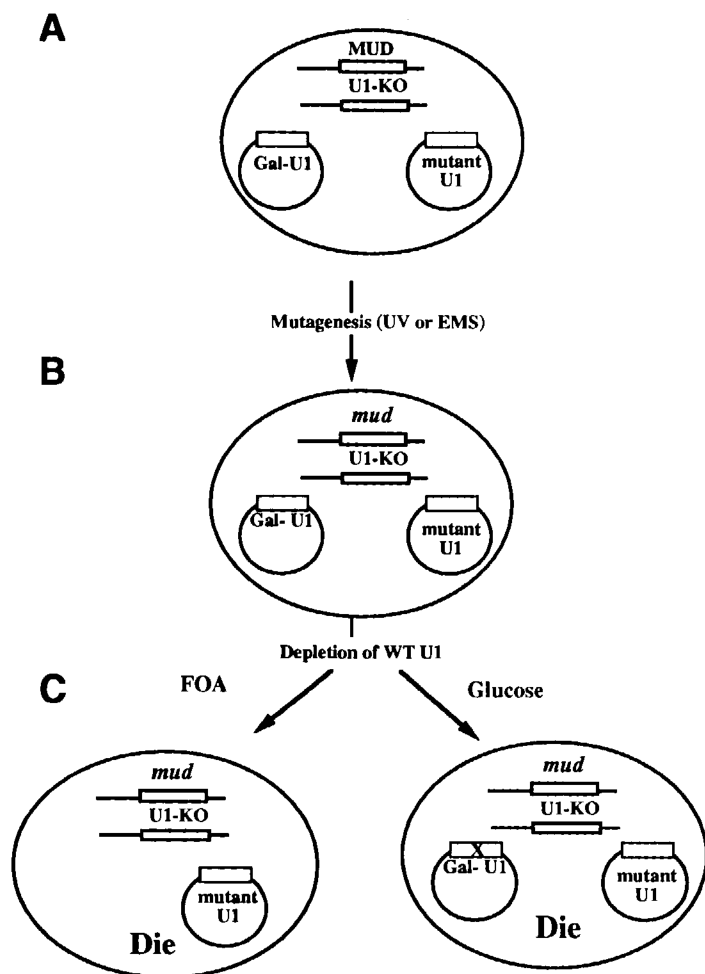

Figure 2. Enhancer screen for mud (mutant U1 die) mutants. (A) The starting strain contains a knock-out of the chromosomal U1 snRNA gene (U1-KO). It also carries a URA3 plasmid expressing wild-type U1 in the presence of galactose but not in the presence of glucose (Gal-U1), and a TRP1 plasmid constitutively expressing a mutant U1 from the U1 promoter (mutant U1). This strain is wild-type for the putative MUD gene(s) (MUD). (B) Mutagenesis of the starting strain results in some mud mutants. (C) mud mutants are identified by depletion of wild-type U1 either by glucose repression of the Gal promoter (as denoted by the × over the Gal-U1 gene), or by growth on 5-FOA which selects against URA3 on the Gal-U1 plasmid. Mutants which die under these conditions are inviable with the mutant U1 and so contain a mud (mutant U1 die) mutation.

Protocol 3. Identification of recessive/dominant mutations

1. Patch the mutants (*Protocol 2*, step 3) onto Trp^- Ura^- 3% galactose/1% sucrose plates. Prepare a lawn of the wild-type strain of opposite mating type on Trp^- Ura^- + 3% galactose/1% sucrose.
2. Mate the patches with the lawn by replica plating to YPD. Incubate overnight.
3. Select for diploids by replica plating on His^- Arg^- + 3% galactose/1% sucrose plates.
4. Replica plate the diploids to 5-FOA plates; diploids with recessive mutations will grow whereas diploids with dominant mutations will not grow.

Protocol 4. Determination of the number of complementation groups

1. Cross the synthetic lethal mutants of opposite mating type to each other. Mate individual lawns of Mat**a** mutants grown on Trp^- Ura^- + 3% galactose/1% sucrose plates with patches of Matα mutants, or vice versa.
2. Select the diploids on His^- Arg^- + 3% galactose/1% sucrose.
3. Replica plate to 5-FOA and YPD. Mutants of the same complementation group are identified by failure to grow on 5-FOA and YPD plates (containing 2% glucose).

Because the U1 synthetic lethal mutant loci are recessive (see Section 2.3), the wild-type genes can be cloned by transforming the mutant hosts with a yeast genomic library (*Protocol 5*).

Protocol 5. Cloning of the mutant genes

Reagents

- Yeast genomic library cloned in the centromeric LEU2 plasmid p366 (P. Hieter)
- PEG–LiTE: dissolve 46.6 g PEG 3350 (Sigma, P4338) in LiTE to make 100 ml of solution (autoclaved or filter sterilized)
- Salmon sperm DNA: 10 mg/ml, freshly boiled and kept on ice
- LiTE: 100 mM lithium acetate, 10 mM Tris pH 7.5, 1 mM EDTA, autoclaved

Method

1. Inoculate the mutant strain in 5–10 ml YPD + 3% galactose/1% sucrose medium. Grow overnight to an A_{600} = 1 (1–2 × 10^7 cells/ml).

Protocol 5. *Continued*

2. Dilute to 2–4 × 10^6 cells/ml in 50–100 ml fresh YPD medium + 3% galactose/1% sucrose, and regrow to 1 × 10^7 cells/ml (two to three generations).
3. Harvest the cells by centrifugation at 2400 *g* for 5 min. Wash the cells by resuspending in 0.1 of the initial culture volume of LiTE. Pellet the cells by centrifuging again.
4. Resuspend cells in LiTE at 2 × 10^9 cells/ml (1/200 of the initial culture volume).
5. Set-up five to ten transformation reactions in separate microcentrifuge (Eppendorf) tubes which contain:
   - 50 μl cells (10^8 cells) in LiTE
   - 1–5 μg yeast genomic library DNA
   - 50 μg of boiled salmon sperm DNA
6. Add 300 μl sterile PEG–LiTE; mix gently.
7. Incubate at 30°C with slow motion for 30–60 min.
8. Heat shock at 42°C for 15 min in a water-bath.
9. Spin for 10 sec in a microcentrifuge. Wash the pellet with 1 ml sterile H_2O, and centrifuge again.
10. Resuspend in 100–200 μl H_2O.
11. Plate one transformation on Leu^- + 3% galactose/1% sucrose to control for transformation efficiency (usually 3–4 × 10^3 transformants/plate); plate the other transformations on Leu^- + 2% glucose plates. Only those transformants able to grow in the absence of the Gal-inducible wild-type U1 snRNA expressed from the URA3 plasmid will form colonies.
12. Grow at 30°C for two to three days. On average, 1/1000 Leu^+ transformants is able to grow on Leu^- + 2% glucose and therefore to rescue the mutation. The rescued transformants also grow on 5-FOA. Both the wild-type U1 gene and the wild-type MUD gene are expected to be able to rescue the mutant hosts (*Figure 2*).

Protocol 6. Recovery of plasmids from yeast

Equipment and reagents

- Extraction buffer: 100 mM NaCl, 10 mM Tris pH 8, 1 mM EDTA, 1% sodium dodecyl sulfate, 2% Triton X-100
- Phenol:chloroform: 50% phenol (equilibrated to pH 8.0), 50% chloroform

- Minimal medium plates: 10.5 g K_2HPO_4, 4.5 g KH_2PO_4, 1 g $(NH_4)_2SO_4$, 0.5 g Na citrate.$2H_2O$. Add one or more of the following as needed: 36 mg Trp, 107 mg Leu, 36 mg Ura. Dissolve in 990 ml H_2O and add 15 g agar. Autoclave and cool to 65°C. Add: 1 ml 1 M $MgSO_4$ (filter sterilized), 0.5 ml 1% thiamine hydrochloride (Vitamin B1), 10 ml 20% glucose (pre-made and sterile). If required, add ampicillin to 100 mg/litre. Stir and pour plates.
- Siliconized glass beads (212–300 microns, Thomas, 566R50). To siliconize the beads, soak them overnight in 1 M HCl. Rinse extensively with deionized water. Mix the beads with tetrachloromethane containing 5% Siliclad (Sigma, D6258). Wash extensively with deionized water. Rinse with 95% ethanol. Bake overnight.

Method

1. Grow 1.5 ml of the rescued mutant strain to saturation in Leu^- + 2% glucose medium.
2. Harvest cells by centrifugation and wash once with water.
3. Add 200 μl glass beads and 250 μl extraction buffer.
4. Vortex at high speed for 2 min.
5. Add 250 μl phenol:chloroform, vortex and centrifuge.
6. Mix the supernatant with 2.5 vol. of ethanol.
7. Incubate on dry ice to precipitate.
8. Centrifuge (e.g. 10 min at top speed in a microcentrifuge) and wash the nucleic acid pellet with 70% ethanol.
9. Resuspend the pellet in 20 μl H_2O.
10. Use 1 μl to transform electrocompetent *E. coli* cells according to standard protocols. The *E. coli* strain MC1066 [D(lac IPOZYA)$_{x74}$, galU, galK, $strA^R$, $hsdR^-$, trpC9830, leuB6, pyrF74::Tn5 (km^R)] is good in selectively recovering yeast plasmids containing the LEU2 (or the TRP1, or the URA3) gene because each of these three yeast genes can complement the corresponding mutant *E. coli* genes.
11. Select the *E. coli* transformants on minimal medium supplemented with 20 mg/ml L-tryptophan, 20 mg/ml uracil, and containing 100 μg/ml ampicillin.

2.3 Results and discussion of U1 snRNA enhancer screen

Our first attempt used a traditional synthetic lethal strategy and is outlined in *Figure 2*. Both UV and EMS mutagenesis were utilized. The starting strain contained the mutant U1 RNA gene on a TRP1 plasmid and a wild-type U1 gene under the control of a galactose-inducible promoter on a URA3 plasmid. Two starting strains were utilized which contain different mutant U1 genes, as depicted in *Figure 1*. One of the mutant genes (ΔYC+LII26A) is missing the yeast core region and has a G to A point mutation in the A loop. The other contains a point mutation at the 5′ end of U1 (U1–4U) (*Figure 1*).

The starting strains were mutagenized with a survival rate of 5–10%; a total of 34 000 viable colonies were screened in both **a** and α mating-type backgrounds and 178 putative 'mud' (mutant U1 die) mutants were identified. Many of them are 'U1-linked', i.e. the lethal phenotype is caused by additional mutations on the starting mutant U1 gene rather than mutations in *trans*-acting factors. These mutants can be identified by plasmid shuffling.

All the mud mutants retained for further study were recessive; they were assigned to complementation groups by crossing the mutants obtained from the two mating types and testing the ability of the diploids to grow on 5-FOA plate, i.e. to grow without wild-type U1 snRNA. If the diploid strain cannot live without wild-type U1 snRNA, the haploid strains from which it is derived are likely to carry mutations at the same locus. Originally, 16 complementation groups were identified (mud11 and mud12 were misidentified in the initial subscreen and were dropped later from the list because they were either U1-linked or had reverted; *Table 1*).

Table 1. Characterization of mud mutants[a]

Strain	Commitment	U1 snRNP	In vivo splicing CUP1 assay	Gene
Wild-type	–	–	–	–
mud1	*	*	*	U1A
mud2	*	–	*	MUD2
mud13	*	–	*	CBP20
mud7-1	*	–	*	PRP5
mud10-1	*	–	*	chr IV/492aa
mud15	–	*	*	NAM8
mud16-1	–	n.d.	–	PRP42
mud5-2	*	n.d.	*	chrXVI/316aa
mud5-3	*	–	*	
mud6-1	–	*	*	SAR1
mud6-2	–	–	n.d.	
mud3-1	*	–	*	
mud3-2	*	–	*	Not clonable
mud4-1	–	*	n.d.	Not clonable
mud4-2	–	–	*	
mud4-3	–	*	n.d.	
mud8-1	*	n.d.	–	Not clonable
mud8-2	*	*	n.d.	
mud9-2	*	*	*	Not clonable
mud14-1	*	n.d.	n.d.	Not clonable

[a] Several assays were used to characterize the mud mutants. Mutant extracts were analysed for commitment complex formation (commitment) in the presence of labelled pre-mRNA by fractionation on native gels. Mutant extracts were analysed for U1snRNP integrity/stability (U1 snRNP) by fractionation on native gels and blotting with U1 snRNA probes. The *in vivo* splicing efficiency was determined by transforming the mutant strains with intron-containing CUP1 reporter constructs and by examining growth on copper-containing plates; copper resistance directly reflects splicing efficiency. * affected; – not affected; n.d. not determined.

Our general strategy has been to subscreen potential splicing mutant strains by biochemical means. Those strains that pass all of the genetic tests *and* show interesting biochemical phenotypes are pursued in more detail. Although these assays are not described here, they can be easily found in the references. The first approach was to make splicing extracts from the mutant strains and assay splicing commitment complexes and spliceosome formation. Three mud mutants were chosen on the basis of their strong *in vitro* complex formation phenotypes. For mud1, the two commitment complexes (cc1 and cc2) migrate faster than those of the wild-type strain (1). For mud2, only one of the commitment complexes, cc2, has an altered mobility. For mud13, commitment complex levels are much lower than those of the wild-type strain, and the mobility of both complexes is markedly decreased. When these three mud mutants were rescued by a wild-type library and the rescuing clones sequenced, it turned out that Mud1p is the yeast homologue of the mammalian U1A protein (1); Mud2p has homology to the mammalian U2AF65 protein (2); and Mud13p corresponds to CBP20, the small subunit of the nuclear cap-binding complex (13). All three of them are implicated in the early steps of splicing by a number of criteria, but only Mud1p is a tightly associated U1 snRNP protein (1, 4).

Before cloning the remaining 11 mud genes, we applied two more assays to see how extensively the mutants affect splicing and/or U1 snRNP structure. One was to fractionate the mutant extracts on a native gel and blot with U1 snRNA-specific probes (14). Since the U1 snRNP from a mud1 extract runs faster on a native gel compared to wild-type (1), this assay can be an indicator of the stability and/or integrity of U1 snRNP. A second assay was to analyse *in vivo* splicing efficiency with several CUP1 reporter genes (15). For this approach, the endogenous CUP1 gene of each mud mutant was deleted and the mud strains transformed with constructs containing the CUP1 gene interrupted by different introns (13). In this assay, the efficiency of *in vivo* splicing is reflected by the strain's ability to grow on copper-containing medium. From our experience, this *in vivo* splicing assay is more reliable, sensitive, and less labour-intensive than the more traditional β-Gal assay (16). The results of these assays are summarized in *Table 1*. Note that most of the mud mutants show splicing phenotypes by these two criteria.

We then attempted to clone all remaining 11 mud mutants with a wild-type library (LEU, CEN). As expected, growth of the mutants was rescued by the wild-type U1 snRNA gene (which represented up to 50% of the rescuing plasmids), and these were eliminated by a PCR reaction using U1 snRNA-specific primers, and the nucleic acid pellets prepared from the rescued yeast strains as described in *Protocol 6*. Only the non-U1 rescuing plasmids were sequenced. Subclones were generated and transformed back into the mutant strain to determine which of the ORFs present in the original rescuing plasmid was responsible for rescuing the synthetic lethal phenotype.

The results indicate that at least four of the remaining 11 mud mutants

correspond to genes functionally related to U1 snRNP or early steps in splicing.

(a) Mud7p is Prp5p, a protein functioning in U2 snRNP addition (17, 18).

(b) Mud10p is a new yeast U1 snRNP protein (Tang *et al.*, unpublished data; R. Lührmann, personal communication).

(c) Mud15p is NAM8, a high copy suppressor of mitochondrial splicing mutations (19). This protein is also a new yeast U1 snRNP protein (Tang *et al.*, unpublished data; R. Lührmann, personal communication).

(d) Mud16p is Prp42p, a recently identified, new yeast U1 snRNP protein (McLean and Rymond, personal communication).

Therefore of the 14 original complementation groups, at least seven are U1 snRNP proteins or collaborate with U1 snRNP during the early steps of splicing complex formation.

The two remaining cloned genes are MUD5 and MUD6. A gene rescuing mud6 encodes Sar1p, which is involved in protein transport from the endoplasmic reticulum to the Golgi apparatus (20). Whether this protein is related to splicing is unknown. At present, it is not even certain that the original synthetic lethal mud6 mutation is a mutant in the SAR1 gene. Another rescuing ORF, MUD5, is without a defined function.

We were unable to clone the remaining five mud mutants; i.e. all the rescuing clones encoded wild-type U1 snRNA. There are two possible explanations for this failure. One is that the wild-type rescuing gene is missing from the library. Since we have not tried other libraries, this possibility cannot be ruled out. Another explanation, more likely in our opinion, is that more than one mutation contributes to the synthetic lethality. In this case, any single wild-type gene will be unable to rescue the phenotype. This explanation is supported by other observations suggesting that the mutagenesis rate (approximately 10% survival) was too high in this first screen. For example, the SAR1 gene that rescues the synthetic lethal phenotype of mud6 is unable to rescue the *in vivo* splicing defect as assayed with the CUP *in vivo* reporter genes (data not shown). It is likely that there is resident in the strain a mutation in addition to sar1 that contributes to the splicing phenotype. The traditional way to solve this kind of problem is to backcross the mutant strain to the wild-type parental strain to segregate the multiple mutations. Unfortunately, this is cumbersome in this particular experimental configuration: to retain the mutant U1 snRNA plasmid for synthetic lethality assays, the diploid has to be grown on minimal medium, which prevents efficient sporulation.

The high mutagenesis rate/multiple mutation problem became more obvious during the execution of our second synthetic lethal screen using the same basic approach (3). In this case, the focus of attention was MUD2, which was identified in the first screen as an inessential gene that collaborates with U1

snRNP during commitment complex formation. A number of arguments suggested that Mud2p interacts with pre-mRNA on the 3′ side of the intron, in the vicinity of the branchpoint sequence. Our interest was also heightened by sequence homology between Mud2p and the important and well studied mammalian splicing factor U2AF65 (2). To identify additional candidates that collaborate with Mud2p and (possibly) with the branchpoint sequence, we carried out a synthetic lethal screen in a genetic background containing a deletion of MUD2.

One candidate mutant, M2S6, contained low levels of U2 snRNA and was also temperature-sensitive (ts) for growth at 37° C. The ts phenotype was rescued by a gene called SNF7, which did not, however, rescue the U2 snRNA phenotype. When M2S6 was crossed to a wild-type strain and tetrads dissected, the low U2 level phenotype and the synthetic lethality co-segregated but not with the temperature sensitivity, i.e. the mutant strain contained at least two mutant genes. A gene encoding the yeast homologue of U2B" protein was cloned by rescuing the synthetic lethality, and this gene is indeed responsible for the U2 snRNA phenotype (3). The altered U2 snRNA levels presumably reflects an effect of the mutant on U2 snRNP assembly or lifetime. The synthetic lethality presumably reflects an interaction between Mud2p and U2 snRNP during the addition of the latter to the commitment complex, similar to the interaction between U2AF65 and U2 snRNP in mammalian spliceosome formation.

3. Identification of synthetic lethal mutations using the red/white sectoring assay

Although the MUD2 synthetic lethal screen described in Section 2.3 identified an important yeast U2 snRNP protein, it did not identify important non-snRNP collaborators of Mud2p. As a consequence, we undertook another synthetic lethal screen focused on the MUD2 protein (5). This third screen was carried out at lower levels of mutagenesis, which necessitates examining a much higher number of colonies. It also utilized the red/white sectoring assay (11). Rather than containing a complete deletion of MUD2, the starting strain expressed the carboxy terminal third of the protein. This portion of Mud2p appears to be the most important part of the protein: it contains a single recognizable domain (an RBD or RNA binding domain), with extensive primary sequence homology to the C terminal RBD of human U2AF65 (which has three RBDs), and it has biological activity, i.e. it is sufficient to rescue the synthetic lethality of a MUD1–MUD2 double knock-out (Abovich, unpublished data). The rationale is that providing the RNA binding region of the protein may focus the screen on factors that interact (directly or indirectly) with Mud2p via protein:protein interactions.

3.1 General reagents and strains

3.1.1 Plasmids

(a) Screening plasmid: 'your favourite gene' (YFG) is cloned into the vector pCH1122 (11, 21). pCH1122 is a centromeric plasmid containing the URA3 and ADE3 markers. In the MUD2 screen, a galactose-inducible version of the MUD2 gene was cloned into the pCH1122 vector (5).

(b) Testing plasmid: YFG is cloned into the centromeric vector pRS315 containing the LEU2 marker. In the MUD2 screen, the testing plasmid contained the complete MUD2 transcription unit.

3.1.2 Yeast strains

(a) CH1305: Mat**a**, ade2, ade3, leu2, ura3, lys2, can1.

(b) CH1462: Matα, ade2, ade3, leu2, ura3, his3.

These two strains have been described in detail (11, 21) and are used to construct the starting strains for the synthetic lethal screen. The ade2 and ade3 mutations are required for the colony white/red sectoring assay. The ura3 mutation allows for the selection of the URA3-containing screening plasmid, and also for selection on 5-FOA plates for loss of the plasmid. The leu2 mutation allows for selection of the testing plasmid.

Strain CH1305 is usually used to carry out the screen because it grows faster than strain CH1462. However performing the screen in both mating types might accelerate the identification of complementation groups since the generated mutants can be crossed right away.

3.1.3 Construction of the starting strains

If it is inessential, disrupt YFG in strain CH1305 (and CH1462) and transform this knock-out strain with the pCH1122-based screening plasmid. If YFG is essential, the purpose of the screen may be to identify mutations synthetic lethal with a conditional allele of YFG; in this case, the wild-type chromosomal copy is replaced by the conditional mutant allele of YFG using standard genetic methods (12). The resulting strain is transformed with the pCH1122-based screening plasmid and transformants are selected on Ura^- + 2% glucose plates. The starting strain does not rely on the screening plasmid to grow and is able to lose the pCH1122-based plasmid when plated on non-selective medium; this plasmid loss (i.e. ADE3 gene loss) is reflected by the appearance of colonies with white sectors when the starting strain is streaked on YPD + 4% glucose (instead of 2%) plates and incubated at 30°C for five to six days. The ability of the starting strain to sector should be established prior to starting the screen.

In the MUD2 synthetic lethal screen we have carried out, the chromosomal wild-type MUD2 gene was replaced by the region encoding just the third RBD of MUD2. This strain was transformed with the plasmid pCH1122-MUD2 encoding wild-type Mud2p (5).

3.1.4 The synthetic lethal screen

The basic idea of the screen is to mutagenize the starting strain containing YFG on the screening plasmid and to isolate mutants for which growth is plasmid-dependent; these mutants appear as solid red colonies (i.e. non-sectoring) and fail to grow when streaked on 5-FOA plates. These candidate mutants are then transformed with the testing plasmid carrying YFG and tested for regained sectoring, which strongly suggests that the mutant depends on YFG (11, 21). Finally, the wild-type copy of the mutant gene is cloned by transforming the mutant strain with a yeast library in a LEU2 vector; these transformants are then selected for the ability to grow on 5-FOA and tested for the restoration of red/white sectoring.

3.1.5 Media and plates

All the media and plates are prepared according to standard protocols (ref. 12, and see Chapter 8). If not specified, 2% glucose is included in media and plates. YPD + 4% glucose (instead of 2%) plates are used for the sectoring assay. Cells are grown at 30°C unless otherwise stated.

3.2 Protocols

Protocol 7. Mutagenesis and identification of candidate synthetic lethal colonies[a]

Equipment and reagents

- See *Protocol 1*

Method

1. Grow the starting strain overnight in 20 ml of Ura^- + 2% glucose medium to an A_{600} of 1–2 (about 2×10^7 cells/ml), and mutagenize by UV irradiation or with EMS as described in *Protocol 1*. Include a non-mutagenized control sample to determine the rate of survival, and determine conditions that yield between 50–70% viable cells (*Table 2*).
2. Dilute and plate the mutagenized cells on YPD + 4% glucose plates (approx. 800 viable mutagenized cells/150 mm diameter plate); incubate at 30°C for five to eight days (in the dark if cells were mutagenized by UV, to avoid photoreversion). If necessary, the cells can be incubated at 25°C, but the process is slower and cells sector less at that temperature.
3. Look for any red non-sectoring colonies; streak candidates for single

Protocol 7. *Continued*

colonies on the same YPD + 4% glucose medium to retest the non-sectoring phenotype. Incubate again at 30 °C or 25 °C for five to eight days. It is not unusual to have as few as 10% of the candidates that retest as non-sectoring.

4. Test the mutants that retested as non-sectoring single colonies on a battery of plates (21):
   (a) Synthetic complete and synthetic complete minus Ura plates to identify any ADE3 convertants that may have already lost the screening plasmid.
   (b) 5-FOA plates to distinguish the following types of mutants:
      (i) True plasmid-dependent candidates which fail to papillate or produce only petite papillae. Both the desired mutants and some background mutants specific to this sectoring screen will behave in this manner.
      (ii) Non-plasmid-dependent mutants which produce large red papillae.
      (iii) ADE3 gene convertants which produce large red papillae.
5. Transform the plasmid-dependent candidates with the testing plasmid alone and the testing plasmid expressing YFG using the transformation procedure described in *Protocol 5*.
6. Select the transformants on Leu⁻ + 2% glucose medium.
7. Streak the Leu⁺ transformants for single colonies on YPD + 4% glucose. If any mutant is dependent on YFG, red/white sectoring or white colonies will appear within four to seven days in the presence of the testing plasmid containing YFG, but not in the presence of the plasmid alone, which serves as a control. The ability of any mutant strain to lose the screening plasmid in the presence of the testing plasmid is the definitive experiment to identify a true plasmid-dependent mutant; this subscreen allows the elimination of mutants that are dependent on ADE3.

[a] A detailed discussion of this genetic approach and additional technical tips have been published by Holm (21).

To analyse the synthetic lethal mutations in a diploid background, the mutant strains (obtained in the Mat**a** CH1305 background) are crossed with the wild-type Matα CH1462 strain containing a disruption of YFG. The MUD2 synthetic lethal mutants were crossed with the CH1462 strain in which the MUD2 gene was replaced by the LEU2 gene (*Protocol 8*).

Protocol 8. Identification of dominant/recessive mutations

1. Streak the mutant strains on a Ura⁻ + 2% glucose (or YPD) plate and the CH1462 derivative on a Leu⁻ + 2% glucose (or YPD) plate.
2. Mate the two types of strains by replica plating to YPD. Incubate overnight.
3. Select for the diploids by replica plating to Ura⁻ Leu⁻ + 2% glucose.
4. Streak the diploids for single colonies on YPD + 4% glucose and 5-FOA plates. Diploids with recessive mutations will sector on YPD and grow on 5-FOA, whereas diploids with dominant mutations will not.

The number of complementation groups represented by the recessive mutants is determined by crossing each mutant to all the other mutants. Tetrad dissection of the diploids described in *Protocol 9* allows the generation of haploid spores which contain the mutation in either mating type.

Protocol 9. Identification of complementation groups

1. Grow the diploids obtained according to *Protocol 8* overnight on YPD plates.
2. Replica plate to potassium acetate plates to induce sporulation. Incubate at 30 °C for two to three days.
3. Tetrad dissect the diploids on YPD according to standard procedures (12). Grow the spores for two to three days.
4. Test the genotype of complete tetrads by replica plating to different drop-out plates.
5. Analyse the segregation of the synthetic lethal mutation by streaking each of the four spores to YPD + 4% glucose and/or to 5-FOA plates. A 2:2 segregation of the mutant phenotype is expected: two sectoring wild-type spores which grow on 5-FOA (sector⁺, FOA⁺) and two non-sectoring mutant spores which do not grow on 5-FOA (sector⁻ and FOA⁻).
6. Determine the mating type of the mutant spores by crossing to Mat**a** and Matα tester strains containing compatible selectable markers. For each mutation, select an **a** and an α spore.
7. Mate the Mat**a** and Matα mutant spores on YPD and select for diploids on 5-FOA plates. Two spores containing non-allelic mutations will complement each other's defect and the diploid will be able to grow on 5-FOA; these mutations fall into different complementation groups. Diploids that fail to grow on 5-FOA contain allelic mutations which belong to the same complementation group.

Protocol 10. Cloning of the mutant genes

The cloning of the genes corresponding to recessive mutations is carried out essentially as described in *Protocol 5*.

Method

1. Transform the mutant strain with a yeast genomic library in the LEU2 centromeric vector p366 (P. Hieter) by following the procedure described in *Protocol 5*. Transform one aliquot of cells with the p366 vector alone as a control for background.
2. Select the transformants on Leu⁻ + 2% glucose plates.
3. Identify the clones that rescue the synthetic lethal phenotype by replica plating the transformants to 5-FOA.
4. Isolate the rescuing LEU2 plasmids from the FOA⁺ colonies according to *Protocol 6*.

3.3 Results and discussion of MUD2 synthetic lethal screen

The screen identified several genes that are candidates for encoding Mud2p interacting proteins (*Table 2*). The post-genetic strategy was identical to that described in Section 2.3, namely, biochemical criteria were used to determine which genes would be pursued in more detail. The results focused on two proteins, Prp40p and BBP, that likely interact with Mud2p during the splicing process (5). BBP is of particular interest, because the experiments suggested that its mammalian orthologue, mBBP (also known as SF1), interacts with U2AF65, the likely mammalian orthologue of Mud2p. It is now of even more interest, as there is evidence that it interacts directly with the branchpoint sequence (22).

Table 2. MUD2 synthetic lethal (msl) screen

	Mutagenesis		
	UV	EMS	
Total colonies screened =	155 000	71 500	Gene
% survival =	66	59	
	msl-1		= PRP40
	msl-2		= ORF with no homology
	msl-3		= Not clonable
	msl-4	= msl-6	= MUD1 (U1A)
	msl-5		= BBP
		msl-7	= Not determined

4. Concluding remarks

A final consideration, one that is perhaps of most interest to someone considering applying synthetic lethal approaches to a range of RNA relevant problems, is the question: 'How well does it work?' The obvious answer from our experience is 'Damn well!' From our large first effort, a conservative estimate is that at least 50% of the genes (seven of 14 complementation groups) encode gene products that collaborate intimately with U1 snRNP during splicing complex formation. The percentage is likely to be higher, as we suspect that some of the genes we have been unable to clone by the most straightforward means also contribute to commitment complex formation (*Table 1*). We do not have a statistically significant sample from our second effort, but the third screen using the red/white sectoring approach also appears to yield a high fraction of U1 snRNP relevant gene products.

Given that the enhancer/suppressor approach is often considered from the pathway point of view, it is perhaps surprising that a substantial fraction of mutant genes do not encode proteins that contribute to later steps in splicing complex formation. Although a conclusion may be premature, it is tempting to speculate that the apparently narrow focus may be a consequence of the single rate-limiting step created by the initial U1 snRNA mutation. This suggests that synthetic lethal genetics may be targeting a single multicomponent complex (the commitment complex in the case of U1 snRNP) rather than the pathway to which this complex contributes. The argument suggests that this application of synthetic lethal genetics can functionally replace complex purification and identification of the subcomponents. If this turns out to be generally true, it means that we have unwittingly accomplished what we set out to do, which was to do biochemistry by doing genetics.

References

1. Liao, X. C., Tang, J., and Rosbash, M. (1993). *Genes Dev.*, **7**, 419.
2. Abovich, N., Liao, X. C., and Rosbash, M. (1994). *Genes Dev.*, **8**, 843.
3. Tang, J., Abovich, N., and Rosbash, M. (1996). *Mol. Cell. Biol.*, **16**, 2787.
4. Tang, J. and Rosbash, M. (1996). *RNA*, **2**, 1058.
5. Abovich, N. and Rosbash, M. (1997). *Cell*, **89**, 403.
6. Miklos, G. L. G. and Rubin, G. M. (1996). *Cell*, **86**, 521.
7. Kretzner, L., Rymond, B. C., and Rosbash, M. (1987). *Cell*, **50**, 593.
8. Kretzner, L., Krol, A., and Rosbash, M. (1990). *Proc. Natl. Acad. Sci. USA*, **87**, 851.
9. Liao, X., Kretzner, L., Séraphin, B., and Rosbash, M. (1990). *Genes Dev.*, **4**, 1766.
10. Gozani, O., Feld, R., and Reed, R. (1996). *Genes Dev.*, **10**, 233.
11. Kranz, J. E. and Holm, C. (1990). *Proc. Natl. Acad. Sci. USA*, **87**, 6629.
12. Guthrie, C. and Fink, G. R. (1991). In *Methods in enzymology*, Vol. 194, (ed. C. Guthrie and G. R. Fink), pp. 389–98, Academic Press.
13. Colot, H. V., Stutz, F., and Rosbash, M. (1996). *Genes Dev.*, **10**, 1699.

14. Séraphin, B. and Rosbash, M. (1989). *Cell*, **59**, 349.
15. Stutz, F. and Rosbash, M. (1994). *EMBO J.*, **13**, 4096.
16. Legrain, P. and Rosbash, M. (1989). *Cell*, **57**, 573.
17. Dalbadie-McFarland, G. and Abelson, J. (1990). *Proc. Natl. Acad. Sci. USA*, **87**, 4236.
18. Ruby, S. W., Chang, T.-H., and Abelson, J. (1993). *Genes Dev.*, **7**, 1909.
19. Ekwall, K., Kermorgant, M., Dujardin, G., Groudinsky, O., and Slonimski, P. P. (1992). *Mol. Gen. Genet.*, **233**, 136.
20. Barlowe, C., d'Enfert, C., and Schekman, R. (1993). *J. Biol. Chem.*, **268**, 873.
21. Holm, C. (1993). *Methods*, **5**, 102.
22. Berglund, J. A., Chua, K., Abovich, N., Reed, R., and Rosbash, M. (1997). *Cell*, **89**, 781.
23. Boeke, J.D., Lacroute, F., and Fink, G.R. (1984). *Mol. Gen. Genet.*, **197**, 345.