Rescuing yeast mutants with human genes

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Abstract

The fission yeast *Schizosaccharomyces pombe* and the budding yeast *Saccharomyces cerevisiae* have, in addition to being extensively studied themselves, both been utilized for the last quarter century as experimental systems for the isolation of genes from other organisms. Mutations conferring growth defects in either of the two yeast strains have frequently been complemented by expression of cDNA libraries from heterologous species, often human. Many successful experiments have utilized available yeast mutations to allow successful complementation by a human gene, which can thus be deduced to have the same, or an overlapping function as the mutated yeast gene. However complementation in yeast has also been used with success to study two fields, apoptosis and steroid receptor signalling, which, at first glance, seem to be foreign to the yeast life cycle.

Keywords: yeast; mutations; complementation; human; cDNAs

INTRODUCTION

Fission yeast (*Schizosaccharomyces pombe*) and budding yeast (*Saccharomyces cerevisiae*) are single cell eukaryotes, which have proved to be extraordinarily amenable to studies in both genetics and biochemistry. Both *S. pombe* and *S. cerevisiae* were in the vanguard of organisms which were subject to complete genome sequencing [1, 2]. Knowledge of the genome sequences has been exploited by the respective user communities to further enhance our understanding of these model eukaryotes. This review addresses the use of the two yeast species by many groups to study the expression of human genes in a clearly defined genetic system. Perhaps surprisingly for single cell organisms, some aspects of complex processes such as apoptosis and steroid receptor signalling have been successfully studied. In this review, we first describe some of the technical requirements necessary to undertake a complementation experiment. Then we give a brief summary of some of the many studies involving complementation of yeast mutants with human genes.

MINIMUM REQUIREMENTS FOR A SUCCESSFUL COMPLEMENTATION EXPERIMENT

To successfully rescue a yeast mutation with a human gene, a number of parameters should be fulfilled. To aid screening there should be a ‘no-growth’ phenotype under clearly defined conditions and the mutation should not show detectable reversion to wild type.

It is important that human coding sequence(s) are presented in a format such that they can be transcribed and translated by the yeast cellular machinery. It is known that both *S. cerevisiae* and *S. pombe* have extremely short introns with mean lengths of 256 and 107 bp, respectively [3]. These figures contrast with an average intron length of ~3300 bp in the human genome [4]. Consequently, both *S. cerevisiae* and *S. pombe* are poor at splicing together human exons. For this reason, human coding sequences whether individual or *en masse* are normally supplied as cDNAs. The consensus sequence immediately preceding the translation initiation codon...
differs between yeast and mammals, although translation in yeast has been shown to be only moderately influenced by sequence context around the AUG [5]. However, it is undoubtedly preferable to utilize a yeast 5’ upstream region where possible.

A brief survey of a sample of the published literature shows that in the early days of this field of study, yeast ‘null’ mutations were frequently temperature sensitive [6] or nonsense alleles with premature stop codons (for example ade2-1, used in [7]). With the advent of PCR and recombination technologies, there has been a distinct switch to recombination-mediated replacement of the target gene with a selectable cassette such as KanMX, for example deletion of the *S. pombe* *ade1* allele [8], or simply dominant functional yeast genes such as the *TRP1* allele [9]. Replacement, rather than inactivation, of the target gene is preferable as there should be no possibility of reversion of the null mutant to wild type.

*Saccharomyces cerevisiae* has roughly 6000 genes of which approximately 1100 are essential for growth of haploid cells under standard laboratory conditions [10]. Essential genes offer both an opportunity and a challenge. Deletion or down-regulation of such a gene will deliver a ‘no-growth’ phenotype which provides an opportunity for rescue by transformation with a human gene or library. The challenge lies in the fact that deletion or down-regulation of an essential gene will result in the death of the cell and thus the end of the experiment. Initially, mutations of essential genes were studied as heterozygous diploids, but this has the problem that any phenotype will be displayed via haploinsufficiency [11]. However, a more promising way to handle essential genes is to make their expression externally regulatable. Thus a yeast strain can be grown up with an essential gene switched on, then transformed with a human gene or library, prior to plating of the transformed cells under conditions where the essential gene is switched off. Hence only yeast cells which have acquired a complementing human sequence will be able to grow. Temperature-sensitive alleles can be used to propagate strains with mutated essential genes [12]. Alternatively a ‘plasmid shuffle’ method can be used, where a wild-type copy of the deleted gene is present on a plasmid with, for example, a *URA3* marker. Once a library has been introduced on a second plasmid, counterselection of the first plasmid is performed using 5-fluoroorotic acid [13].

In search of a more systematic approach, three groups have described collections of down-regulatable essential genes, in each case utilizing the tetracycline-regulatable (tetO) promoter [14]. The EUROFAN II consortium produced 99 promoter-substitution strains, regulated by the tetO7 promoter (http://web.uni-frankfurt.de/fb15/mikro/euroscarf/data/ess.html). A collection of 602 strains each with a tetO7 promoter substitution was produced by a North American consortium [10]. The third collection comprises 173 strains with tetO2 promoter substitution produced by a UK collaboration [15]. The latter collection deliberately utilized tetO2 rather than tetO7, after making the observation that the tetO7 promoter can be activated by at least one human transcription factor [15, 16], thus raising the possibility that a human gene appearing to complement a down-regulated essential yeast gene could do so by up-regulating expression of the yeast gene rather than providing a complementary function. Part of the collection of 173 tetO2 promoter-substitution strains had previously been used in a study to isolate human cDNAs by functional complementation using entire human cDNA libraries provided in a yeast expression vector [17]. Twenty-five tetO2 or tetO7 (EUROFAN II) replacement strains were screened, resulting in the isolation of six human cDNAs. In all six instances, when expression of both the endogenous yeast gene and the human cDNA was switched off, the yeast cells ceased to grow. Thus these six human cDNAs appear to provide genuine complementation. The reasons for this rather low success rate of ~25% are unclear. Human cDNA libraries containing approximately 750,000 recombinants were constructed in yeast expression vectors from kidney and cerebellum. Both libraries underwent size selection during construction to eliminate fragments of <800 bp. The yeast vector used, pMETtPGK3-1/2/3, comprised a family of three otherwise identical vectors each with an ATG in a different reading frame relative to the cloning site. Thus it seems unlikely that the low rate of complementation is solely due to lack of human sequence complexity.

It has previously been observed that in many instances, promoter-substitution strains exhibit either no, or at best slight, growth defects when plated under non-permissive conditions [15]. This may be due to the fact that down-regulation of a gene should result in cessation of synthesis of the corresponding mRNA, but will not result in depletion
of existing amounts of stored protein or mRNA. A study has been published where individual yeast genes are modified such that the N-terminal of the encoded protein is fused to a heat-inducible degron cassette [18]. When cells are grown at 37°C the cassette unfolds, exposing lysine residues that are sites of ubiquitylation. When Ubr1 protein is co-expressed in the same cell, the targeted protein is degraded. This system was shown to work efficiently and a collection of 108 degron-tagged essential \textit{S. cerevisiae} genes was described, of these ~60% show a Ubr1-induced temperature-sensitive phenotype.

When expressing human genes or libraries in a yeast cell, there is a choice between using a constitutive or an inducible promoter. Use of an inducible promoter, such as \textit{GAL4} or \textit{MET3} allows a greater degree of overall control. In particular, switching off expression of the human gene should result in a 'no-growth' phenotype, and this has been shown to have great utility in identifying false positives [17]. Several different human cDNA libraries have been constructed in yeast expression vectors. These include a collection of 33,000 cDNA clones from the human hepatoma cell line HepG2, these sequences being under the control of the constitutive glyceraldehyde-3-phosphate dehydrogenase promoter [7]. As mentioned above, two large libraries each containing \(7.5 \times 10^5\) independent clones were constructed from human kidney and cerebellum: these are in the expression vector pMETtPGK3-1/2/3, which has a repressible \textit{MET3} promoter [19]. More recently 26,000 human clones from the Mammalian Gene Collection [20] were retrofitting into pMET3tPGK3-1/2/3 by PCR amplification of individual clones followed by bulk insertion into the yeast vector (M.J. Osborn and J.R. Miller, unpublished data). A library containing \(1 \times 10^5\) primary transformants was produced. Use of this library in future yeast–human complementation projects should allow access to human transcripts which are low copy number and may never have been previously sampled in complementation experiments. It should be noted that a report indicated, that in at least one case, presence of a human 5’ untranslated region upstream of a coding sequence led to inefficient translation in \textit{S. cerevisiae} [21]. Thus it may prove difficult or impossible to design and construct the perfect human cDNA library for complementation in yeast.

**RESCUE OF YEAST MUTATIONS WITH HUMAN GENES**

One of the very first instances of a human gene being used to complement a yeast mutation is also one of the most famous studies in this field. Lee and Nurse [6] used a temperature-sensitive mutation in the \textit{S. pombe} \textit{cdc2} gene to isolate the human orthologue by complementation. The \textit{cdc2} gene encodes a cyclin-dependent protein kinase. The temperature-sensitive \textit{S. pombe} mutation used was unable to proceed beyond the G1 and G2 checkpoints of the cell cycle. Lee and Nurse thus transformed the \textit{cdc2} temperature-sensitive mutant with a human cDNA library provided in an SV40 expression vector. Advantageously the SV40 early promoter was known to function in \textit{S. pombe}. Transformants, selected by co-transformation of a plasmid containing a prototrophic marker, were initially grown at the permissive temperature, and then switched to the non-permissive temperature. Of the small number of colonies obtained, two yielded plasmids which, when purified were identical and encoded the human \textit{CDC2} gene. This experiment had therefore elegantly demonstrated that complementation of yeast mutations could be used to isolate human genes. Furthermore, the observation that a human protein could perform a role in the \textit{S. pombe} cell cycle suggested that all or parts of the underlying mechanism were conserved between the two species. Subsequent studies have shown that other human cell division cycle genes have yeast orthologues. However, in at least two cases it has been demonstrated that functional complementation of a yeast mutation can only be achieved by use of a chimeric protein comprising the N-terminus of the normal yeast protein followed by the C-terminus of the human homologue. The \textit{S. cerevisiae} \textit{PRP17/CDC40} gene that encodes a protein containing seven WD motifs [22] is involved in both the splicing reaction and the cell division cycle. A human EST was identified bioinformatically which coded for a protein closely related to yeast Prp17/Cdc40. The human protein had a divergent N-terminal but possessed a C-terminal containing seven WD motifs which was highly similar to the yeast protein. Complementation of yeast cells containing a deletion of the \textit{PRP17/CDC40} gene with the human cDNA did not yield viable colonies. However, a chimeric protein containing the divergent yeast N-terminus
and the seven WD motif-containing human C terminus did complement the yeast mutation [22]. A similar result was earlier obtained for another spliceosomal protein Prp16 [23]. As above, a human orthologue with divergent N-terminus and similar C-terminus was isolated. Complementation of a yeast PRP16 knockout was not achieved by the human cDNA. Once again, a chimeric molecule containing the yeast N-terminus joined to the human C-terminus did yield complementation. In both cases, the necessity for the yeast N-terminus is thought to be due to human–yeast sequence divergence in this area, the human protein may thus be unable to interact with necessary partners within the yeast splicing or cell division machinery.

More than one yeast gene can be deleted and complemented by human cDNAs as shown with the MEX67/MTR2 double-knockout strain where the human TAP-p15 complex replaces an essential conserved role in nuclear export [24].

HOW MUCH PROTEIN SEQUENCE SIMILARITY IS REQUIRED FOR COMPLEMENTATION?
Subunit h is a component of the S. cerevisiae ATP synthase F0 and is essential for growth on non-fermentable carbon sources such as glycerol. Hence a yeast strain deleted for subunit h will not grow on plates containing glycerol as the sole carbon source. However, growth was obtained on glycerol when this deletion strain was transformed with a plasmid encoding bovine coupling Factor 6 [25]. Biochemical studies showed that bovine Factor 6 (F6) does interact directly with yeast ATP synthase in the subunit h deletion mutant. This result was very surprising as the two proteins show an amino acid identity level of merely 14.5%, far below what was previously thought to be necessary for functional complementation.

EXPRESSION OF THE PROAPOPTOTIC BAX PROTEIN IN YEAST
Saccharomyces cerevisiae is known to be susceptible to cell death in response to a range of stimuli which have previously been shown to cause apoptosis in mammalian cells, such as the presence of free radicals or osmotic stress. Saccharomyces cerevisiae does possess orthologues of mammalian apoptotic proteins, for example the caspase-like molecule Yca1, which appears to mediate the apoptotic response to hydrogen peroxide treatment [26] via autoactivation induced by cleavage at an active-site cysteine. However, S. cerevisiae does not appear to possess any representatives of the Bcl-2 family of pro- and antiapoptotic proteins. Nonetheless, it has been known for more than a decade that expression of the mammalian proapoptotic protein Bax causes cell death in S. cerevisiae [27, 28]. This was shown to be a specific process related to apoptosis as Bax expression will not kill cells which lack mitochondria [28], furthermore co-expression of either Bcl-2 or Bcl-XL can rescue yeast from Bax-mediated death. Bax expression was subsequently shown to cause release of cytochrome c from mitochondria to cytosol [29]. Bax expression was also shown to be lethal in S. pombe [30]. These observations provided the background for genome-wide screens for Bax suppressors where yeast was transfected with both an inducible Bax gene and an entire cDNA library, provided on a suitable expression vector. The assay was simply to find surviving colonies after Bax induction. This procedure has been carried out many times in different laboratories [31], yielding a variety of genes whose expression will suppress Bax-induced lethality. These include: Bax-Inhibitor 1 (BI-1) [27] an antiapoptotic protein found to be conserved in both animals and plants [32]; sphingomyelin synthase 1, an enzyme that synthesizes sphingomyelin from the proapoptotic substrate ceramide [33]; Ku70, a protein which appears to block endogenously expressed Bax from translocating to the yeast mitochondria [34]; HMGB1, an HMG protein highly expressed in human breast cancer cells [35]. HMGB1 was shown in mammalian cells to inhibit apoptosis induced by a number of different agents including treatment with UV, CD95L and TRAIL. The utility of using a genetically tractable organism such as S. cerevisiae in apoptosis research is illustrated by the recent debate as to whether the mitochondrial protein EndoG is actually an apoptotic effector. Although originally described as such, for example [36], two different groups reported production of EndoG knockout mice which did not exhibit apoptotic defects [37, 38]. However, subsequent work in S. cerevisiae [39] shows that when mitochondria are undergoing respiration, deletion of NUCP1
(the yeast orthologue of EndoG) decreases apoptotic death. Thus, in yeast at least Nuc1p/EndoG has a pro-death role.

**STEROID RECEPTOR SIGNALLING PATHWAYS**

*Saccharomyces cerevisiae* does not encode proteins functioning as steroid receptors, however several studies have shown that by expression of appropriate receptors, steroid signalling pathways can be assembled. This field has previously been reviewed [40, 41], so this section will be confined to describing an important experiment which attempted to identify factors which can impact upon the glucocorticoid receptor (GR) signal transduction pathway.

In this work [42], a yeast strain was constructed containing first a GR expression vector and second a glucocorticoid response element (GRE) controlling expression of both the *HIS3* gene and the *lacZ* gene. This strain was shown to grow in the absence of histidine when 1 \( \mu \text{M} \) deoxycorticosterone, a GR ligand, was supplied. In contrast, the same strain fails to grow on media lacking histidine when the GR ligand is 300 nM dexamethasone (Dex). These observations formed the basis of an unbiased screen to identify factors affecting GR signalling. The yeast strain was mutagenized (UV or EMS treatment) then plated on histidine-minus media in the presence of 300 nM Dex. Previously no growth had been observed, but after mutagenesis 156 mutant colonies were obtained, of which 100 showed both increased GR activity and \( \beta \)-gal activity in response to the ligand. Two mutants, both recessive, were selected for further study. Dose–response assays showed that one mutant termed *lem3* (ligand–effect modulator) showed a large increase in both GR potency and efficacy; the other mutant, *lem4*, showed increased ligand potency but efficacy (maximum activation observed with saturating levels of ligand) was the same as the parent strain. Experiments involving GR truncations were then pursued which showed that *lem3* and *lem4* affect different GR functions. Simplistically, the glucocorticoid receptor comprises (i) an N-terminal regulatory domain, (ii) a DNA binding and nuclear localization domain, (iii) a C-terminal hormone binding and signalling domain. Truncation of the N-terminus, removing the regulatory domain, had no effect in *lem4* cells, but there was no or little activity, as measured by \( \beta \)-gal readout, in *lem3* cells. In contrast, removal of the C-terminal hormone-binding domain caused a large but not total decrease in GR function in *lem4* cells, whilst having no effect in *lem3* cells. The apparent requirement for the presence of the ligand-binding domain for the *lem4* phenotype was confirmed by showing that there was an 8- to 10-fold increase in binding of radioactive dexamethasone in *lem4* as opposed to wild-type cells. What did the *lem3* and *lem4* genes encode? This question was answered by exploiting the previous observation that both mutations were recessive. *Lem3* and *lem4* were inserted independently into a haploid yeast strain which contained the GR and two integrated GR-sensitive reporters, *lacZ* and *CAN1*. The latter is a yeast arginine permease, which allows intake of canavanine, a toxic arginine analogue. Thus whilst cells lacking the *lem* mutants will not respond to the GR-ligand dexamethasone and hence will grow in the presence of canavanine, *lem3* and *lem4* mutants will fail to grow when canavanine is added to the medium. The *lem3* and *lem4* mutants were each transformed with a low copy number yeast genomic library and plated on Dex + canavanine. Subsequent analysis showed that *lem3* was complemented by YNL323w and that *lem4* was complemented by YML008c. *Lem3* encodes a protein of unknown function, thought to be located in the plasma membrane and ER; *lem4* encodes ERG6, a methyltransferase converting zymosterol to fecosterol in the biosynthesis of ergosterol. Confirmation that YNL323w and Erg6 were respectively responsible for the *lem3* and *lem4* mutations was obtained by disrupting both genes in a wild-type background and showing that the two deletion strains had increased responsiveness to Dex. A *lem3*, *erg6* double–deletion mutant was also constructed and tested; there was an additive phenotype in response to Dex, indicating that each mutant is located in a different pathway. Thus this work led to the isolation of two genes located on independent pathways which appear to down-regulate glucocorticoid receptor function. As noted by the authors, neither of these genes would have been predicted on a ‘candidate’ basis to modulate GR activity. Thus use of yeast, an organism where GR signalling can be reconstituted, appears to have been fully justified.

**DICER**

The nuclease Dicer is a key component of the RNAi pathway. Dicer cleaves dsRNA into 21–23 nt long
small interfering RNAs. These siRNAs bind PAZ Piwi Domain (PPD) proteins to form ribonucleoprotein complexes termed RISC (RNA-induced silencing complexes) [43] which base-pair to specific mRNAs and thus mediate their destruction. Database searching with the sequence of human Dicer indicates that there is a Dicer orthologue in *S. pombe*, but perhaps surprisingly, not in *S. cerevisiae* [8]. Deletion of *S. pombe* Dicer (*dcr1*) caused a range of effects included slowed growth on both solid and liquid media, lagging of chromosomes during anaphase and failure to suppress transcription from centromeric repeat sequences. The Dicer enzyme from wild-type *S. pombe* was shown to be capable of producing characteristic 23 nt fragments from a variety of longer ds-RNA templates. When *dcr1* was deleted, *S. pombe* was shown to undergo slow growth on medium containing thiamine and thiabendazole. Expression of human Dicer from an episomal vector gave a partial relief of this phenotype. Thus the human enzyme shares at least some of the function(s) of the yeast enzyme [8].

*Schizosaccharomyces pombe* possesses a solitary member of the PPD family, *ago1*. Deletion of this gene showed that it was not essential for haploid growth; however deletants were less efficient at septum formation and showed a higher percentage of bi-nucleated cells than wild type, suggesting a role for PPD proteins in cell cycle regulation [43]. These authors also demonstrated that expression of hAgo2, a human PPD protein, could correct the defect in cytokinesis caused by *ago1* deletion. This suggests that PPD proteins may play a role in regulation of the mammalian cell cycle.

HUMAN BLOOM SYNDROME GENE AND DNA REPLICATION

The BLM protein is a member of the RecQ family of human DNA helicases, which when mutated, is responsible for Bloom syndrome, an autosomal recessive disorder characterized by predisposition to malignancy and chromosomal instability. The BLM gene has a homologue termed *SGS1* in *S. cerevisiae*. The Sgs1 protein is also a helicase which acts as a suppressor of illegitimate recombination [9]. Human BLM has been shown to suppress the resulting increased homologous and illegitimate recombination in a yeast *SGS1* disruption mutant [9]. Sgs1 also interacts with other DNA helicases including Dna2, which is thought to be involved in maturation of Okazaki fragments. The temperature-sensitive growth defect of a yeast DNA2 mutant was suppressed by human and Xenopus DNA2 genes and also by hBLM. An hBLM mutant protein, with a single amino acid substitution causing an inactive DNA helicase, failed to complement the yeast DNA2 mutant, indicating that helicase activity is required for complementation [44]. DNA2 has been shown to interact with Fen1, a 5’ to 3’ exonuclease required for Okazaki fragment processing and maturation. Co-immunoprecipitation was used to show interaction between BLM and scFen1 when co-expressed in yeast [44]. All this evidence suggested that BLM can function at the replication fork in yeast. The brief description provided here of this work, serves to illustrate the universality of the proteins involved in control of DNA recombination and hence, genome stability.

IN THE REVERSE DIRECTION

The kinetochore, the location for attachment of spindle microtubules to the chromosome, is a structure composed of many proteins, located on the mitotic chromosome and adhering to the centromere. Many, but not all, mammalian kinetochore proteins show significant sequence homology to those found in yeast. Relationships between homology and function were analysed for two human proteins: the inner kinetochore protein CENP-A and the microtubule-binding protein CLIP-170 [45]. Both human proteins have yeast homologues, human CENP-A is related to yeast Cse4 and human CLIP-70 is the homologue of yeast Bik1. Stable human cell lines expressing each human and yeast protein as GST fusions were obtained, all four fusion proteins were shown to be correctly localized: GFP-CENP-A and GFP-Cse4 to the centromere; GFP-CLIP-170 and GFP-Bik1 to the kinetochore. Knockdown of CENP-A or CLIP-170 by RNAi-mediated depletion resulted in observable mitotic phenotypes: CENP-A depletion resulted in small aberrant nuclei and lagging chromosomes in mitotic cells; CLIP-170 depletion resulted in small aberrant nuclei and lagging chromosomes in mitotic cells; CLIP-170 depletion resulted in mitotic cells accumulating lagging chromosomes and to a very large increase in numbers of mitotic as opposed to interphase cells relative to a mock transfected control. Both knockdowns caused a large decrease in numbers of living cells after 72 h with RNAi. These observations paved the way to ask whether
each yeast gene could substitute for its human homologue in RNAi-treated cells.

Both yeast genes were supplied as GFP fusion proteins. It was found that Cse4 could rescue cells undergoing knockdown of CENP-A, however Bik1 expression failed to rescue cells with depleted CLIP-170. Curiously, it had previously been shown that expression of CENP-A in S. cerevisiae deleted for Cse4 did not provide functional complementation. Thus this study demonstrated that whilst centromeric proteins are highly conserved across evolution, the ability to function correctly may be dependent on subtle sequence components.

CONCLUSIONS
A large number of published studies have reported the successful use of both budding and fission yeast as vehicles for isolation of human genes by complementation. Parts of complex systems, such as steroid receptor signalling and apoptosis, have been assembled in yeast. A note of caution should be sounded, in that there have undoubtedly been many unsuccessful attempts to complement yeast mutations with human genes. As these studies are largely unreported, it is difficult to assign technical reasons for their failure. As mentioned earlier, the precise sequence requirements for best expression of a human cDNA in a yeast cell are still unclear. It should also be noted that whilst no expression will result in non-complementation, over-expression or a lack of control of expression, may prove deleterious or lethal and again result in non-complementation.

Key Points
- Complementation of yeast mutations has been frequently used as a tool to isolate human genes.
- A number of suitable high-quality human cDNA libraries have been prepared in yeast expression vectors.
- Processes such as steroid receptor signalling and Bax/Bcl-2 interactions can be reconstituted in yeast.
- Expression of a large number of essential yeast genes can be manipulated in either promoter substitution strains or degron-tagged coding sequence strains.
- Precise sequence requirements for efficient expression of a human gene in yeast are still unclear, this caveat particularly applies to the 5’ untranslated region and sequence around the initiation codon.

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