

Prion protein gene polymorphisms in *Saccharomyces cerevisiae*

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Summary

The yeast *Saccharomyces cerevisiae* genome encodes several proteins that, in laboratory strains, can take up a stable, transmissible prion form. In each case, this requires the Asn/Gln-rich prion-forming domain (PrD) of the protein to be intact. In order to further understand the evolutionary significance of this unusual property, we have examined four different prion genes and their corresponding PrDs, from a number of naturally occurring strains of *S. cerevisiae*. In 4 of the 16 strains studied we identified a new allele of the *SUP35* gene (*SUP35Δ19*) that contains a 19-amino-acid deletion within the N-terminal PrD, a deletion that eliminates the prion property of Sup35p. In these strains a second prion gene, *RNQ1*, was found to be highly polymorphic, with eight different *RNQ1* alleles detected in the six diploid strains studied. In contrast, for one other prion gene (*URE2*) and the sequence of the *NEW1* gene encoding a PrD, no significant degree of DNA polymorphism was detected. Analysis of the naturally occurring alleles of *RNQ1* and *SUP35* indicated that the various polymorphisms identified were associated with DNA tandem repeats (6, 12, 33, 42 or 57 bp) within the coding sequences. The expansion and contraction of DNA repeats within the *RNQ1* gene may provide an evolutionary mechanism that can ensure rapid change between the [*PRION*⁺] and [*prion*⁻] states.

Introduction

The yeast *Saccharomyces cerevisiae* encodes several functionally distinct proteins that can act as novel epigenetic determinants through a self-propagating change in their conformation. By analogy with the mammalian PrP

protein, implicated as the self-propagating infectious agent in a number of neurodegenerative diseases such as Bovine Spongiform Encephalopathy (Prusiner *et al.*, 1998), the yeast proteins have been referred to as prions (Wickner, 1994). Perhaps the best characterized yeast prion protein is Sup35p, the determinant of the [*PSI*⁺] prion. Sup35p is a component of the yeast translation termination factor (Stansfield *et al.*, 1995; Zhouravleva *et al.*, 1995). In a [*PSI*⁺] cell, a large proportion of Sup35p appears to be functionally inactive due to aggregation of its prion form (Patino *et al.*, 1996; Paushkin *et al.*, 1996). This leads to a defect in translation termination that can be assayed as enhanced suppression of nonsense mutations in either the *ADE1* gene (UGA) or the *ADE2* gene (UAA) (Serio and Lindquist, 1999).

The Sup35p prion protein is modular in both its structure and function. The C-terminal region (aa 254–685) carries out the essential function of the protein in translation termination (Ter-Avanesyan *et al.*, 1993; 1994), whereas the extreme amino terminus of Sup35p (aa 1–123) defines the prion-forming domain (PrD) that is responsible for the prion-like behaviour of Sup35p but is not essential for Sup35p function *per se* (Ter-Avanesyan *et al.*, 1994; Tuite, 2000). These two regions are separated by the highly charged M (middle) region (aa 124–253) that may contribute to the prion-like properties of this protein (Liu *et al.*, 2002). A striking feature of the Sup35p-PrD is the presence of two sequence elements both of which are crucial for the conversion to, and maintenance of, the prion form of Sup35p; a Gln- and Asn-rich region between amino acids 8 and 33 (DePace *et al.*, 1998) and five complete (and one partial) copies of an oligopeptide (8–9 aa) repeat (Tuite, 2000). Deletion of one or more of the full repeats (designated R1–R5) prevents the resulting mutant Sup35p from maintaining the [*PSI*⁺] determinant in the absence of wild-type Sup35p, although the mutant Sup35p molecules are still able to take up the prion state in the presence of wild-type Sup35p (Parham *et al.*, 2001). The partial repeat (R6) is dispensable for [*PSI*⁺] propagation (Parham *et al.*, 2001).

Although the amino acid sequence of the Sup35p-C-terminal region is highly conserved from yeast to man (Inagaki and Doolittle, 2000), the Sup35p-PrD exhibits a high level of divergence in amino acid sequence with significant divergence between relatively closely related

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yeast species (Inagaki and Doolittle, 2000; Nakayashiki *et al.*, 2001). Although several of the fungal Sup35p N-terminal regions have the potential to drive protein aggregation in *S. cerevisiae* (Chernoff *et al.*, 2000; Kushnirov *et al.*, 2000; Santoso *et al.*, 2000), only in the case of *Kluyveromyces lactis* has this property been demonstrated in the natural host species (Nakayashiki *et al.*, 2001). A search for the aggregated prion form of Sup35p in several natural isolates and industrial strains of *S. cerevisiae* revealed that the strains examined were [psi⁻] (Chernoff *et al.*, 2000). This raises the issue of whether or not [PSI⁺] is a laboratory acquired trait arising as a consequence of long-term growth in a non-native environment.

In addition to Sup35p/[PSI⁺], two other yeast proteins with prion-like properties have been identified in *S. cerevisiae*: Ure2p/[URE3] (Wickner, 1994) and Rnq1p/[RNQ⁺] (Sondheimer and Lindquist, 2000). Each contains regions with the characteristic features of the Sup35p-PrD, namely a high Gln and/or Asn content and few charged residues. A third protein, New1p, also has a PrD (Santoso *et al.*, 2000) although it remains to be established whether the New1p protein *per se* is a prion. Neither the function of Rnq1p or New1p has been identified apart from their prion-like properties nor has a phenotype been associated with the presence of the respective prion form. [RNQ⁺] – unlike [URE3] and [PSI⁺] – is commonly found in many laboratory strains (Sondheimer and Lindquist, 2000).

To gain a fuller understanding of the evolutionary importance of yeast prions and their prion-forming domains requires a detailed analysis of the structure and behaviour of these proteins in non-laboratory strains of *S. cerevisiae*. Jensen *et al.* (2001) have recently reported the results of a sequence survey of the Sup35p PrDM region (aa1–253)

from 23 strains of *S. cerevisiae*, over half of which were clinical isolates. Intriguingly, they only identified three alleles with single non-synonymous amino acid substitutions in the Sup35p-PrD and only one of these (a Q30R substitution) was within the region we have defined as required for PrD activity (Parham *et al.*, 2001). The aim of our study was to extend this approach to other prion genes and in so doing have discovered novel polymorphisms in both the *SUP35* and *RNQ1* genes that can be attributed to deletion or expansion of short (<50 bp) tandem nucleotide repeats within the coding sequences of these genes.

Results

Identification of a novel SUP35 allele in clinical isolates of S. cerevisiae

In order to explore the frequency with which both non-synonymous and synonymous nucleotide substitutions occur within the PrDM-encoding region of the *SUP35* gene, this region was amplified by high fidelity PCR from 16 different clinical isolates of *S. cerevisiae* (SCI1–16; Table 1) and both strands sequenced. The Sup35p-PrD from four strains (SCI3, SCI4, SCI7 and SCI11) were found to have an identical 19 aa deletion when compared with the Sup35p-PrD sequence from the other 12 strains examined and to the two originally reported *SUP35* gene sequences from laboratory strains (Accession no. M21129, Kushnirov *et al.*, 1988; X07163, Wilson and Culbertson, 1988). We designate this allele *SUP35Δ19*. The deletion, spanning amino acids 59–77, removed one complete oligopeptide repeat (R3) and parts of the two flanking repeats R2 and R4 (Fig. 1) with the resulting PrD only carrying three intact oligopeptide repeats. A number

Table 1. Clinical isolates of *Saccharomyces cerevisiae* used in this study.

| Strain | Clinical source | Clinical isolate no. | GenBank Accession no. ^a |
|------------------------|---|----------------------|------------------------------------|
| J941082 ^b | Vaginal, patient recently treated clotrimazole, Belgium | SCI1 | AY028644 |
| J940915 ^b | Vaginal, patient recently treated econazole, Belgium | SCI2 | AY028645 |
| J941047 ^b | Oral, gynaecology patient, Belgium | SCI3 | AY028646 |
| J940610 ^b | Oral swab, gynaecology patient, Belgium | SCI4 | AY028647 |
| J940557 ^b | Vaginal, symptom-less patient, age 38, Belgium | SCI5 | AY028648 |
| J940421 ^b | Oral, AIDS patient, Germany | SCI6 | AY028649 |
| NCPF 8145 ^c | Pleural effusion | SCI7 | AY028650 |
| NCPF 8184 ^c | Wound | SCI8 | AY028651 |
| NCPF 8185 ^c | Blood culture | SCI9 | AY028652 |
| NCPF 8186 ^c | Pancreatic drain | SCI10 | AY028653 |
| NCPF 8290 ^c | Serum | SCI11 | AY028654 |
| NCPF 8313 ^c | Blood culture | SCI12 | AY028655 |
| NCPF 8328 ^c | Peritoneal fluid | SCI13 | AY028656 |
| NCPF 8348 ^c | Blood culture | SCI14 | AY028657 |
| NCPF 8363 ^c | Pus bile duct | SCI15 | AY028658 |
| NCPF 8372 ^c | Prosthetic aortic graft | SCI16 | AY028659 |

a. *SUP35* DNA sequences from the given strain deposited in the GenBank database.

b. From Janssen Research Foundation (Dr Frank C Odds).

c. From National Collection of Pathogenic Fungi.

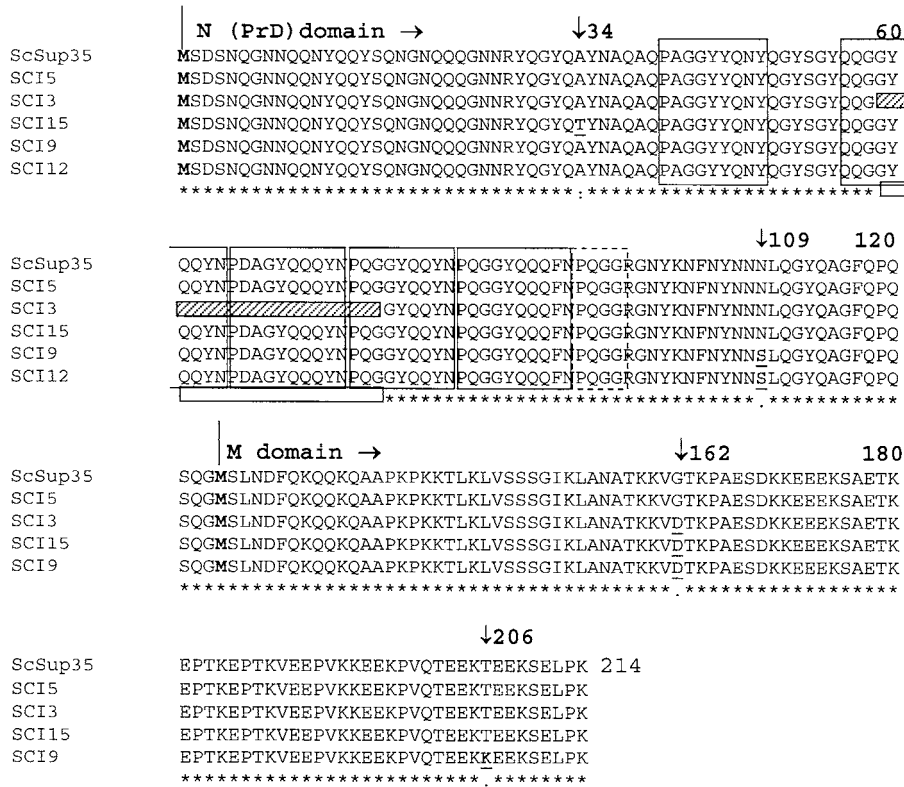


Fig. 1. Amino acid sequence alignment of Sup35p (amino acid 1–214) encoded by five different naturally occurring *SUP35* alleles of *Saccharomyces cerevisiae*. The upper sequence (ScSup35) is that originally reported by Kushnirov *et al.* (1988) and Wilson and Culbertson (1988). Amino acid differences are underlined and their position indicated by the downward arrow (positions 34, 109, 162 and 206). The five complete (R1–R5) and the one partial oligopeptide repeat (R6) are boxed. The deletion found in the SCI3 and SCI4 strains is indicated by the hatched box. The Met residue that defines the start of the M domain (at residue 124) is indicated.

of single amino acid differences in the Sup35p PrDM region were also identified outside the oligopeptide repeat-containing region. For example, all strains, apart from SCI3, had Gly162 rather than Asp162 within the M domain, while SCI15 had an Ala33 rather than Thr33 in the Sup35p-PrD. Strain SCI9 contained two polymorphisms, one in the PrD at residue 109 (Asn to Ser), the other in the M domain at residue 206 (Thr to Lys). A random mutagenesis study of the Sup35p-PrD by DePace *et al.* (1998) did not identify residue 34 as important for $[PSI^+]$ propagation while residue 109 is in the region we have shown to be non-essential for $[PSI^+]$ propagation (Parham *et al.*, 2001). A total of six different *SUP35* alleles were detected amongst the 16 strains studied (Fig. 1).

The complete nucleotide sequence of the *SUP35* gene was determined for four of the strains (SCI2, 3, 4 and 6) with several nucleotide differences being identified in the C domain, although only one of these (in strain SCI3) resulted in an amino acid difference (Q658H). All of the strains – with the exception of SCI9 – could be induced to sporulate although we obtained no evidence for heterozygosity at the *SUP35* locus in any of these strains.

The $[PSI^-]$ status of the naturally occurring strains of *S. cerevisiae*

To confirm the predicted $[psi^-]$ status of the clinical strains homozygous for the *SUP35Δ19* allele we determined the relative distribution of Sup35p between soluble and aggregated forms in two of these strains, namely SCI3 and SCI4. In both strains, Sup35p was soluble to the same extent as a laboratory $[psi^-]$ strain, whereas no soluble Sup35p was detected in the control $[PSI^+]$ strain (Fig. 2). We also determined the subcellular distribution of Sup35p in seven other clinical strains (SCI1, 2, 5–7, 11 and 16); all showed identical patterns to those of SCI3 and 4 (data not shown).

To confirm the deduced $[psi^-]$ status of the strains carrying the *SUP35Δ19* allele, a genetic analysis was undertaken. After sporulation of the SCI3 and SCI4 strains, two haploid spores from each diploid were mated with a $[psi^-]$ *ade2-1 SUQ5* strain. Knowing that $[PSI^+]$ is a cytoplasmically inherited genetic trait (Cox, 1965), the haploid segregants from such a cross would be expected to give rise to 2 red:2 white (2R:2W) spore clones for each tetrad if the strains were $[psi^-]$. In contrast, if they were $[PSI^+]$, one would expect a range of spore phenotypes with indi-

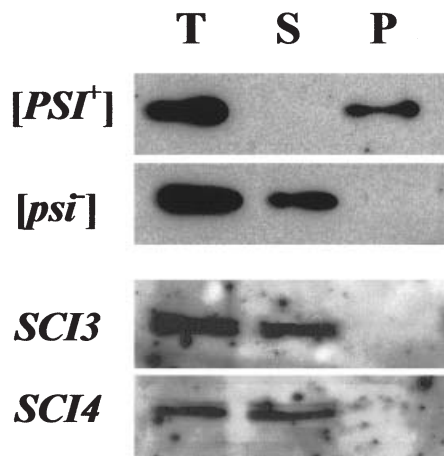


Fig. 2. Western blot analysis of the soluble fraction prepared from cell-free lysates of clinical isolates of *S. cerevisiae* (SCI3 and 4). Cell-free extracts were prepared from two clinical strains, SCI3 and SCI4 and fractionated, as described in *Experimental procedures*, into total (T), soluble (S) and pellet (P) fractions. Following SDS-PAGE using 10% acrylamide gels, protein samples were transferred to nitrocellulose and the Sup35p protein detected using an anti-*S. cerevisiae* Sup35p polyclonal antibody as described in *Experimental procedures*. Control $[PSI^+]$ and $[psi^-]$ 74D-694 strains were also used and the samples are indicated $[PSI^+]$ and $[psi^-]$ respectively. Note that no Sup35p is detected in the soluble fraction from the $[PSI^+]$ strain.

vidual asci showing either 2R:2W, 1R:3W or 0R:4W. In total, 26 tetrads were analysed from such crosses involving two independent SCI3 spores (2c and 5b) and 16 tetrads involving two independent SCI4 spores (3b and 7b). The spore segregation results obtained for both strains clearly indicated that the haploid spores were $[psi^-]$ with a 2R:2W segregation pattern. Poor spore viabilities (<20%) among the other three strains (SCI2, SCI5 and SCI6) meant that statistically significant segregation data could not be obtained.

Oligopeptide repeat deletions in the Sup35p-PrD give rise to a dominant antisuppressor phenotype because the mutant Sup35p is unable to interact with the wild-type Sup35p to form prion aggregates and is therefore available to interact with eRF1 (Sup45p) to form an active eRF

complex (Ter-Avanesyan *et al.*, 1994; DePace *et al.*, 1998; Parham *et al.*, 2001). However, such strains retain 'cryptic' $[PSI^+]$ seeds composed of the wild-type Sup35p (Parham *et al.*, 2001). To evaluate whether Sup35p encoded by the *SUP35Δ19* allele behaved similarly, we exploited a plasmid-based assay that allowed us to test the consequences of Sup35p-PrD manipulations on $[PSI^+]$ maintenance and transmission (Parham *et al.*, 2001).

The PrD-encoding region (aa 1–114) of *SUP35Δ19* was amplified by PCR from the SCI3 and SCI4 strains and inserted in frame with the wild-type MC domain-encoding region of *SUP35* to generate the plasmids pUKC1512-SCI3 and pUKC1512-SCI4 respectively. These two plasmids were individually transformed into the $[PSI^+]$ strain MT700/9d which carries both a disruption of the chromosomal *SUP35* locus (*sup35::kanMX*) and the *URA3*-based plasmid pYK810 carrying a copy of the wild-type *SUP35* gene (Parham *et al.*, 2001). After selection on 5-FOA the $[PSI^+]$ phenotype of the 5-FOA-resistant (i.e. lacking the pYK810 plasmid) transformants was determined. For both pUKC1512-SCI3 and pUKC1512-SCI4, the resulting strains had the red *Ade⁻* $[psi^-]$ phenotype (Fig. 3).

To determine whether the mutant Sup35p encoded by the *SUP35Δ19* allele was a dominant 'Psi No More' (PNM) mutant, i.e. eliminated the $[PSI^+]$ prion, the $[PSI^+]$ strain BSC783/4a was transformed with either pUKC1512-SCI3 or pUKC1512-SCI4. The resulting transformants were red *Ade⁻*, but returned to white *Ade⁺*, i.e. $[PSI^+]$, after loss of the plasmid after extensive growth of the transformants on non-selective medium. These data show that the natural truncated Sup35p variant encoded by the *SUP35Δ19* allele is not 'curing' $[PSI^+]$ from the strain, but rather is an ASU ('antisuppressor') allele as defined by DePace *et al.* (1998).

Polymorphisms in three other yeast prion genes: URE2, RNQ1 and NEW1

The finding of naturally occurring polymorphisms in the *SUP35* gene that inactivated its prion-like behaviour lead

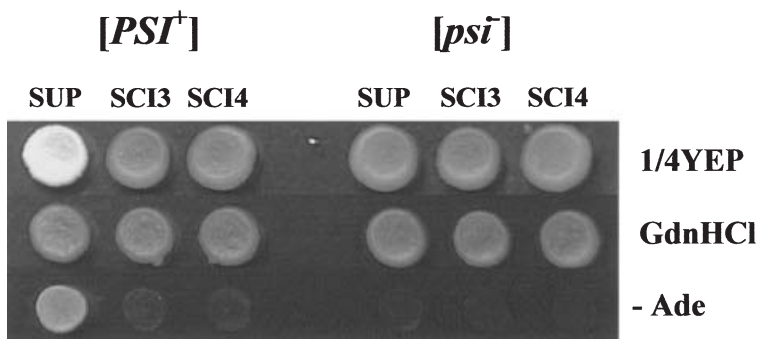


Fig. 3. The Sup35p-PrD encoded by the *SUP35Δ19* is unable to maintain $[PSI^+]$ in the absence of wild-type Sup35p. The plasmid pUKC1512 encoding Sup35p with either the wild-type N domain (aa 1–114) sequence (designated SUP) or the corresponding region from the *SUP35Δ19* allele from either the strain SCI3 or SCI4 was shuffled into either a $[PSI^+]$ or a $[psi^-]$ derivative of MT700/9d as described in the text. After 5FOA selection, individual transformants were spotted onto either rich medium (1/4 YEPD), rich medium containing 3 mM guanidine hydrochloride (GdnHCl) or onto defined medium lacking adenine (–Ade). The results shown are after incubation at 30°C for 4 days.

us to determine whether the other three genes of *S. cerevisiae*, known to encode prion proteins, were polymorphic. Using high fidelity PCR we amplified and sequenced the PrD-encoding sequences from the *RNQ1* (Sondheimer and Lindquist, 2000), *NEW1* (Santoso *et al.*, 2000) and *URE2* (Wickner, 1994) genes from six of the clinical isolates of *S. cerevisiae*, including two of the strains homozygous for the *SUP35Δ19* allele.

Neither non-synonymous nor synonymous substitutions were found in the PrD-encoding region of *URE2* (amino acids 1–65, Masison and Wickner, 1995) when the deduced sequences were compared with the previously reported *URE2* gene sequence (*URE2*/YNL229C Accession no. M35268). Alignment of the six deduced New1p-PrD encoding sequences (amino acids 1–153; Santoso *et al.*, 2000), from the same six strains, with the previously reported *NEW1* gene sequence (*NEW1*/YPL226W, Accession no. NC001148) revealed several nucleotide sequence differences between the different *NEW1* sequences within this region. Only one of these was a non-synonymous substitution; in the strain SCI16 an A to G change at nucleotide 208 of the open reading frame (ORF) results in Asp70 instead of Asn70. No heterozygosity, at either the *URE2* or *NEW1* loci, was observed in these strains.

Comparison of the C-terminally located Rnq1p-PrD-encoding region (amino acid 153–405; Sondheimer and

Lindquist, 2000) from the same six wild-type strains, with the published *RNQ1* sequence (*RNQ1*/YCL028W Accession no. NC001135) indicated a much greater number of polymorphisms at this locus within the PrD-encoding region. Initially, sequence data were generated for the PCR-amplified *RNQ1*-PrD region from the six original diploid strains. Three of the strains (SCI3,4 and 10) gave unambiguous DNA sequencing results whereas DNA sequence analysis of PCR products from three other strains (SCI7, SCI9 and SCI11) gave ambiguous sequences indicating heterozygosity at the *RNQ1* locus in these three strains. For two of these latter strains (SCI7 and SCI11) we were able to generate several haploid spores whereas SCI9 was asporogenous. We independently cloned the different *RNQ1* alleles from SCI7 and SCI11 spores after high fidelity PCR amplification from the original diploid strain.

Eight different *RNQ1* alleles (designated A–H) were identified among the six diploid strains studied (Fig. 4). We consistently found two silent nucleotide substitutions compared with the published *RNQ1* sequence (*RNQ1*/YCL028W Accession no. NC001135); a T instead of C at position 930, and C instead of T at position 1098. Four of the six strains had *RNQ1* alleles with His360 (CAC) rather than the published Gln360 (CAG). The *RNQ1* allele in SCI4 (allele D) had an additional amino acid substitution:

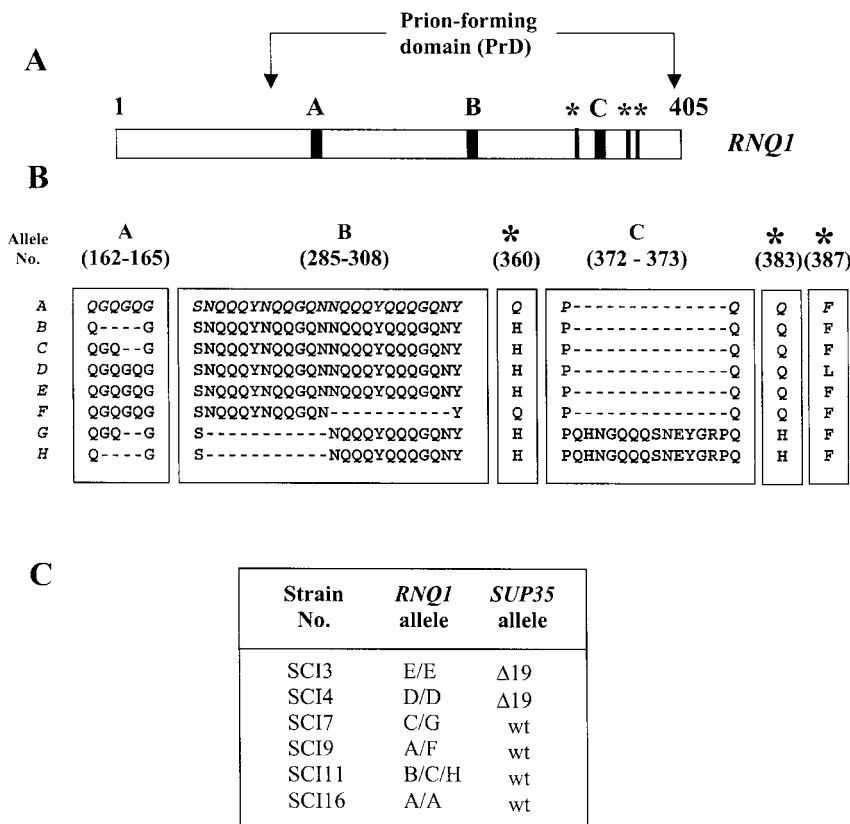


Fig. 4. The polymorphisms in the sequence of the Rnq1p protein detected in six different clinical isolates of *S. cerevisiae*.

A. Schematic representation of the *RNQ1* coding sequence indicating the location of the prion-forming domain (PrD; aa153–405) as defined by Sondheimer and Lindquist (2000), and the location of the various polymorphic regions (A, B, C) and the positions of the single amino acid polymorphisms (*).

B. Amino acid sequence polymorphisms detected in the eight different *RNQ1* alleles (A–H). The amino acid positions are indicated in brackets. Allele A is the sequence originally deposited in the GenBank database under Accession no. NC001135 A – indicates a missing residue compared with the GenBank sequence.

C. A summary of the *RNQ1* and *SUP35* alleles present in the six different strains. Note that strain SCI11 has three alleles most probably because the strain is trisomic for chromosome III which carries the *RNQ1* gene.

Leu387 (TTA) rather than the published Phe387 (TTC) sequence. The asporogenous strain SCI9 was heteroallelic at the *RNQ1* locus with one allele (allele F) containing a 33 bp deletion leading to a deletion of 11 amino acid between residues 297–307 (Fig. 4). Sequence analysis of the *RNQ1* alleles in the other two heteroallelic strains SCI7 and SCI11 identified a variety of additional polymorphisms including 6, 12 and 33 bp in frame deletions and a 42 bp in frame insertion relative to the published *RNQ1* sequence (Fig. 4). The various deletions/insertions mapped to one of three distinct regions (designated A, B and C on Fig. 4) with regions B and C being within the Rnq1p-PrD as defined by Sondheimer and Lindquist (2000). Intriguingly, the strain SCI11 appeared to contain three different *RNQ1* alleles (alleles B, C and H; Fig. 4) indicating that this strain was either triploid or trisomic for chromosome III. These polymorphisms clearly generated Rnq1p protein molecules of different lengths and with different Asn + Gln content in the Rnq1p-PrD, changes which could impair the formation of the $[RNQ^+]$ prion in these strains.

Maintenance of the $[RNQ^+]$ determinant in strains carrying *RNQ1* polymorphisms

With such a degree of polymorphism within the Rnq1p-PrD it was important to determine the $[RNQ]$ status of the haploid strains carrying the various *RNQ1* alleles derived from strains SCI7 and SCI11. To do this we relied on Rnq1p sedimentation analysis (Sondheimer and Lindquist, 2000). All of the haploid strains derived from SCI7 or 11 were $[rnq^-]$ with the majority of the Rnq1p being in the soluble fraction (Fig. 5). However, both the SCI3 and SCI4 $[psi^-]$ strains which carry the wild-type *RNQ1* gene (allele A; Fig. 4) but have the *SUP35Δ19* allele, were $[RNQ^+]$. The subcellular distribution of Sup35p was also examined in these same strains and in all cases was consistent with the strain being $[psi^-]$ (Fig. 2; data not shown).

The PrD-encoding regions of yeast prion protein-encoding genes contain tandemly repeated nucleotide sequences

The detection of a common length polymorphism within the oligopeptide repeat-containing region on the *S. cerevisiae* *SUP35Δ19* allele lead us to ask whether the deletion was related to deletion of an underlying DNA repeat within this region. An analysis of the Sup35p-PrD coding sequence, using the 'Tandem Repeats Finder' program (Benson, 1999), identified a tandemly repeated 57 bp sequence containing 10/57 nucleotide mismatches (Fig. 6). The 19-amino-acid deletion in the truncated Sup35p encoded by the *SUP35Δ19* allele corresponded with the first of these repeats (Fig. 6). Therefore, there is a previously undescribed 57 bp (19 amino acid) duplication in the *SUP35* gene within the PrD-encoding region. No such nucleotide repeats were evident in the M or C regions.

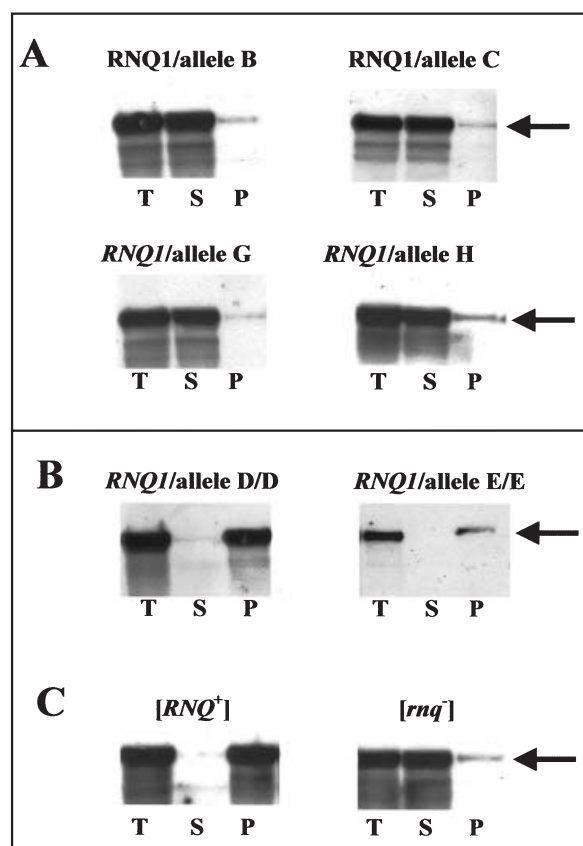


Fig. 5. Determining the solubility status of the Rnq1p protein in various clinical isolates of *S. cerevisiae*. Western blot analysis of various clinical strains of *S. cerevisiae* using an anti-Rnq1p polyclonal antibody is shown. For each strain total cell-free extracts (T) were fractionated into soluble (S) and pellet (P) fractions as described in *Experimental procedures*.

A. Haploid strains carrying the indicated *RNQ1* allele.
B. Diploid strains homozygous for either allele D or E of *RNQ1*.
C. Control $[RNQ^+]$ and $[rnq^-]$ strains. The position of the Rnq1p protein is indicated by the arrow.

A similar analysis of the PrD-encoding regions of the *URE2*, *RNQ1* and *NEW1* genes revealed the existence of a number of other repeated DNA sequences ranging in size from 6 to 42 nucleotides, excluding trinucleotide repeats (Table 2). Significantly, the various polymorphisms identified in the *RNQ1* gene were all associated with repeated nucleotide sequences (Table 2). The *URE2* gene, which showed no polymorphism in the strains we examined, had no nucleotide repeats other than 18 tandemly repeated Asn codons (AAT/C).

Discussion

Evolutionary significance of *SUP35* gene polymorphisms

Comparisons between homologues from a range of eukaryotic species has revealed that the N-terminal Sup35p-PrD is highly variable both in amino acid compo-

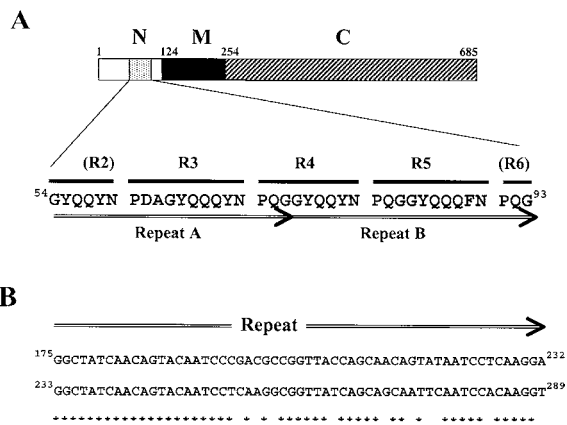


Fig. 6. The *S. cerevisiae* Sup35p-PrD-encoding region contains two copies of a 57 base pair (19 amino acid) repeat one of which is deleted in the *SUP35Δ19* allele.

A. The amino acid sequence of the region of the Sup35p-PrD that encompasses the two copies of the 19 amino acid repeat, i.e. aa 54–93. The repeated peptide sequence is indicated by the arrows (Repeat A and Repeat B). The *SUP35Δ19* allele is missing Repeat A. The positions of the oligopeptide repeats R2 to R6 are indicated. **B.** The DNA sequence of the 57 base pair repeats corresponding to the 19 amino acid repeats. The identical residues are indicated by a *.

sition and length (Inagaki and Doolittle, 2000; Nakayashiki *et al.*, 2001) whereas the essential C-terminal domain shows a much higher degree of cross-species amino acid sequence identity. Only one reported Sup35p sequence lacks such a variable N domain, namely that of the *Giardia lamblia*, an 'early diverging eukaryote' (Inagaki and Doolittle, 2000). We report here the discovery of a novel, naturally occurring *SUP35* allele *SUP35Δ19* that encodes a Sup35p protein lacking 19 amino acids within the oli-

gopeptide repeat-carrying region of the PrD. The resulting Sup35p contains only three rather than the usual five copies of the oligopeptide repeat in the Sup35p-PrD. These repeats, which are related in sequence to the 'octarepeats' present in the mammalian prion protein PrP (Tuite, 1994), are important for the maintenance of $[PSI^+]$ (Ter-Avanesyan *et al.*, 1994; Liu and Lindquist, 1999; Parham *et al.*, 2001).

The truncated Sup35p encoded by the *SUP35Δ19* allele gives rise to an 'anti-suppressor' (ASU) phenotype (DePace *et al.*, 1998) as the *SUP35⁺/SUP35Δ19* heterozygote produces significant levels of soluble Sup35p that is able to participate in translation termination, but also contains a number of cryptic $[PSI^+]$ seeds. Therefore, in our small survey, 25% of the 'wild-type' strains of *S. cerevisiae* examined are unable to form the $[PSI^+]$ prion although a *SUP35/SUP35Δ19* heterozygote should be able to maintain cryptic prion seeds that can allow (re)establishment of the $[PSI^+]$ prion when the *SUP35Δ19* allele is lost. None of the naturally occurring strains we examined were heteroallelic at the *SUP35* locus.

That the *SUP35Δ19* allele appears to be relatively frequent in natural isolates might imply that a selective pressure may exist that selects cells that are not able to establish and maintain the $[PSI^+]$ state in certain ecological niches; for example, in the human bloodstream which is presumably an atypical environment to this species. A previous survey of 13 clinical isolates found only two naturally occurring polymorphic amino acids in the Sup35p-PrD (Jensen *et al.*, 2001), neither of which would be expected to impair $[PSI^+]$ propagation. It remains to be established if the various *SUP35* polymorphisms so far

Table 2. Tandemly repeated DNA sequences in the *URE2*, *RNQ1* and *NEW1* genes.

| Gene | Repeated sequence | Position ^a |
|-------------|---|-----------------------|
| <i>URE2</i> | (AAT) ₅ | 130–144 |
| <i>NEW1</i> | TACAACAAT TACAACAAC TACAACAAC TACAACAAT TATAACAAC TACAATAAC TATAATAAA | 217–279 |
| <i>RNQ1</i> | (A) CAAGGT CAGGGA CAAGGT CAAGGT CAAGGT CAAGGT CAAGGT CAAGGT CAAGGT CAAGGT CAAGGT CAAGGT CAAGGT | 457–516 |
| | (B) CAACCAACAGCAATACAATCAACAAGGCCAAAA CAACCAGCAGCAATACCAGCAACAAGGCCAAAA | 855–920 |
| | (C) AATGAGTATGGTAGACCGCAACAGAATGGTCAACAGCAATCC AATGAGTACGGAAGACCGCAATACGGCGGAAACCAGAATCC | 1057–1140 |

a. The nucleotide position of the repeated sequences given relative to the A of the ATG (initiation codon) of the coding sequence as +1.

described occur in strains isolated from different niches as to date only one non-laboratory strain has been studied and this had no polymorphisms in the Sup35p-PrD (Jensen *et al.*, 2001).

While the function of the N-terminal Sup35p PrD is relatively well established, the role of the non-essential M domain is far from clear. It is a highly charged region of the protein molecule that may act as a flexible linker between the N and C domains (Serio and Lindquist, 1999). We have identified two non-synonymous substitutions in the M domains (Fig. 1), but it remains to be established if these mutations influence the prion-like behaviour of the Sup35p. Gross changes to the M domain of Sup35p can certainly influence the propagation of the $[PSI^+]$ prion determinant although derivatives of Sup35p lacking the M domain can exist in the prion form (Liu *et al.*, 2002).

Within the four *SUP35* C-terminal domains sequenced, only one non-synonymous substitution was found; a Q658H substitution in the SCI3 strain. Jensen *et al.* (2001) only found one non-synonymous nucleotide polymorphism in the C domain among the 23 strains they studied. These data suggest that the essential functional C domain of Sup35p is under different selective pressures to the non-essential (for viability) PrD and M domains.

The RNQ1 prion gene is highly polymorphic

Our analysis of the *RNQ1* gene from six different clinical isolates of *S. cerevisiae* identified eight different *RNQ1* alleles (Fig. 4). Three of the strains were heteroallelic for this locus on chromosome III with one strain (SCI11) apparently trisomic for chromosome III. The product of this gene, the Rnq1p protein, gives rise to the transmissible $[RNQ^+]$ determinant that is present in most laboratory strains of *S. cerevisiae* (Sondheimer and Lindquist, 2000). To date, however, no biochemical function has been assigned to the Rnq1p protein although cells that are $[RNQ^+]$ show a higher rate of *de novo* appearance of several other yeast prion determinants (Derkatch *et al.*, 2001; Osherovich and Weissman, 2001).

In haploid strains derived from two of the clinical strains examined, i.e. SCI7 and SCI11, Rnq1p was mostly present in the soluble fraction indicating these strains were $[rnq^-]$ (Fig. 5). It was noticeable that the four different *RNQ1* alleles in these strains contained deletions of either GQ (alleles C, G) or GQGQ (alleles B, H) between residues 162–165 when compared with the originally reported *RNQ1* allele (Fig. 4). Furthermore, *RNQ1* alleles G and H contained both an 11-amino-acid deletion between residues 286–296, and an insertion of 14 amino acids between residues 372 and 373. In all cases these variations occur within the Rnq1p-PrD as defined by Sondheimer and Lindquist (2000). The two clinical strains which contained *RNQ1* alleles without such changes, i.e. SCI3

(allele E/E) and SCI4 (allele D), were both $[RNQ^+]$ (Fig. 5), although intriguingly both were homozygous for the *SUP35Δ19* allele.

That both the $[RNQ^+]$ and $[PSI^+]$ prion determinants can be stably maintained in the same cell (Derkatch *et al.*, 2001; Osherovich and Weissman, 2001) shows that having two different prion determinants in the same host cell is not necessarily detrimental to the cell. This is exemplified by the study of Derkatch *et al.* (2001) who showed that Rnq1p is the predominant determinant of $[PIN^+]$, a cytoplasmically located prion-like determinant necessary for the *de novo* induction of $[PSI^+]$. These workers, together with Osherovich and Weissman (2001), have also shown that overexpression of other Asn/Gln-rich proteins, e.g. Ure2p and New1p, can mimic the $[PIN^+]$ phenotype. Therefore, the frequency with which a yeast prion determinant appears *de novo* in a cell may be significantly increased if that cell is already $[RNQ^+]$. $[rnq^-]$ strains such as SCI7 and SCI11 may have a much reduced rate of *de novo* conversion of other proteins, such as Sup35p and Ure2p, to their transmissible prion form. However, Bradley *et al.* (2002) have recently shown that $[PSI^+]$ inhibits the appearance of $[URE3]$ suggesting that heterologous prion/prion interactions can either drive or inhibit *de novo* prion conversion in the yeast cell.

Role of tandem repeats in evolution of prion genes/proteins

The two prion genes which showed the most significant degree of polymorphism among the natural isolates we studied (i.e. *SUP35* and *RNQ1*) both contain tandemly repeated nucleotide sequences in their PrD-encoding regions (Table 2 and Fig. 6). Significantly, the polymorphisms we identified in these two genes were generated by changes in numbers of copies of these tandemly repeated sequences. For example, the *SUP35Δ19* allele presumably arose as a consequence of unequal crossing over between the two 57 bp (19aa) repeats (Fig. 6) although we did not find a *SUP35* allele with the reciprocal of such an event, i.e. carrying three copies of the 57 bp repeat. Such alleles, were they to be found, would be of considerable interest as Liu and Lindquist (1999) have provided evidence that increasing the numbers of oligopeptide repeats within the Sup35p PrD leads to a 1000-fold increase in the *de novo* appearance of $[PSI^+]$. The failure to find such an allele could again be taken as evidence for a selective pressure against an ability to efficiently switch to the $[PSI^+]$ state in clinical strains of *S. cerevisiae* although greater numbers of strains need to be analysed before a firm conclusion can be reached. A search for DNA repeats in other available fungal *SUP35* gene homologues revealed a variety of tandemly repeated DNA repeats within the putative N-terminal PrD regions

of these proteins, all of which were multiples of three nucleotides (C. G. Resende and M. F. Tuite, unpubl. data). We have also recently reported the existence of naturally occurring length polymorphisms within the N domain of the *Candida albicans* Sup35p homologue (CaSup35p; Resende *et al.*, 2002) although in this case the polymorphisms appear to result from expansion (or contraction) of a much smaller trinucleotide repeat (CAA) leading to changes in the number of tandem polyglutamine residues. However, this trinucleotide (or the related CAG) may be particularly susceptible to deletion or amplification; for example expansion of polyglutamine tracts within the human Huntingtin gene is associated with an increased propensity to protein aggregation and concomitant neurodegeneration (Usdin and Grabczyk, 2000). In contrast, we did not uncover any *S. cerevisiae* URE2 alleles, in our limited analysis, with changes in the number of Asn residues in the Ure2p-PrD, although the longest poly Asn tract in the Ure2p protein is only 7 of which 5 are encoded by the AAT codon.

There are numerous examples of short tandem DNA repeats in eukaryotic genomes. Such repeats display high rates of polymerase slippage during replication (Strand *et al.*, 1993) or DNA recombination events between multiple loci consisting of homologous repeat motifs (Jankowski *et al.*, 2000) resulting in either expansions or contractions of the repeat number. Tandem DNA repeat instabilities have also been reported in *S. cerevisiae*; for example, Freudenreich *et al.* (1997; 1998) and Maurer *et al.* (1996) have shown that CAG repeat contraction can occur during mitosis while both contraction and expansion can occur during meiosis (Schweitzer *et al.*, 2001). Larger DNA repeats (e.g. 36 bp) also show expansions and contractions in meiosis (Paques *et al.*, 2001). Studies in yeast have shown that trinucleotide repeats are clustered in regulatory genes and primarily located in non-essential regions of the proteins indicating a possible novel form of gene regulation with important consequences for evolution by acting as a source of genetic variation (Young *et al.*, 2000).

Internal protein repeats are found in about 17% of all yeast proteins whereas the frequency is much lower in prokaryotes. This has led Marcotte *et al.* (1998) to propose that proteins containing such repeats may evolve at a faster rate than those that do not. This would mean that proteins with internal repeats would facilitate faster adaptation to changing environments. In bacteria, there are several well-documented examples of genes in which tandem DNA repeat expansion or contraction within coding sequences result in alteration in the nature and hence function of the gene product, e.g. the *Opa* genes of *Neisseria gonorrhoeae* and the LPS genes of *Haemophilus influenzae* (Moxon *et al.*, 1994). Why might there be a need for rapid evolution of the sequence of the Rnq1p

protein? The central importance of the prion form of Rnq1p in the *de novo* conversion of other yeast prions (Derkatch *et al.*, 2001; Osheroich and Weissman, 2001) might provide the clue. The potential for rapid evolutionary changes to the Rnq1p-PrD would in turn alter the frequency at which other prions could potentially raise *de novo* in the population. As suggested by True and Lindquist (2000) the ready emergence of prions such as [PSI⁺] may play an important role in the 'evolvability' of yeast by facilitating adaptation to changes in the cell's environment without necessarily that change in environment triggering the prion conversion. A detailed analysis of the prion-like properties of the Rnq1p variants we have described here, together with a study of the mitotic and meiotic stability of the identified tandem DNA repeats, will be required to support this hypothesis.

A link between prion gene polymorphism and *de novo* prion conversion may not be restricted to yeast prions. The mammalian prion protein gene (*Prnp*) contains a 24 bp repeat region coding the octapeptide repeat, a sequence that bears some resemblance to the oligopeptide repeat in Sup35p (Parham *et al.*, 2001). A number of polymorphisms have been identified in *Prnp* that involve this repeated sequence (Palmer and Collinge, 1993). The deletion of PrP octapeptide repeats, while not eliminating disease in experimental models, does lead to a reduction in the severity of the associated neuropathology consistent with a reduced rate of conversion (Flechsig *et al.*, 2000). However, *Prnp* alleles with an expansion in the number of these repeats is associated with some inherited forms of prion diseases, including Kuru, Creutzfeldt–Jakob disease (CJD), and Gerstmann–Sträussler Scheinker syndrome (GSS) (Palmer and Collinge, 1993; Vital *et al.*, 1999), i.e. these individuals show a higher rate of *de novo* conversion of PrP^C to the disease-associated PrP^{Sc} form.

Experimental procedures

Saccharomyces cerevisiae strains

[PSI⁺] and [psi⁻] derivatives of the strain BSC783/4a (*MAT α* *SUQ5 ade2-1_{UAA} his3-11,-15 ura3-1 leu2-3,-112*; Doel *et al.*, 1994) were used as control strains in the subcellular fractionation and analysis of the yeast cell lysates. BSC783/4a [psi⁻] and BSC783/4c [psi⁻] (*MAT α* *SUQ5 ade2-1_{UAA} his3-11,-15 ura3-1 leu2-3,-112*) were used in the genetic studies to determine the [PSI] status of the *S. cerevisiae* clinical isolates as described below. The strain 74-D690 [PSI⁺] (*MAT α* *ade1-14_{UGA} his3-200 leu2-3,-112 trp1-289 ura3-52*; Chernoff *et al.*, 1995) was used in the genetic studies for evaluating the capacity of *S. cerevisiae* clinical isolates to maintain [PSI⁺]. Strain MT700/9d (*MAT α* , *sup35::kanMX4, SUQ5, ade2-1_{UAA}, his3-11,-15, ura3-1, leu2-3,-112*) transformed with pYK810 (a centromeric plasmid-containing *SUP35*; Kikuchi *et al.*, 1988) was used in prion propagation assays (Parham *et al.*, 2001).

Six clinical isolates of *S. cerevisiae* were obtained from Professor Frank C. Odds (Janssen Research Foundation; Table 1). All six strains gave an assimilation pattern on an API ID32C test confirming, with better than 98% probability, that these were strains of *S. cerevisiae*. Ten different isolates of *S. cerevisiae* from a variety of clinical sources (Table 1) were provided by Dr Patrick Dorr (Pfizer Global Research) and were originally obtained from the UK National Collection of Pathogenic Fungi (NCPF).

Genetic techniques

Standard yeast media, cultivation procedures and genetic techniques were used (Kaiser *et al.*, 1994). Tetrad dissection was performed on a Singer MSM System Micromanipulator. The clinical isolates of *S. cerevisiae* were sporulated on a standard nitrogen-depleted medium (SMA; 0.25% yeast extract, 1% potassium acetate, 0.05% glucose, 0.2% adenine) and the resulting tetrads dissected on to YEPD. [*rho*⁻] strains were generated by growth in the presence of ethidium bromide (10 µg ml⁻¹) on solid YEPD medium and identified by their inability to grow on a non-fermentable carbon source (YEPG; 1% yeast extract, 2% bactopectone, 2% glycerol).

The [*rho*⁻] derivatives of the haploid *S. cerevisiae* clinical isolates were mated with either the BSC783/4a [*psi*⁻] or BSC783/4c [*psi*⁻] strains and the resulting diploids selected on minimal medium containing 2% glycerol as the sole carbon source. A second passage of the diploids on minimal medium containing glycerol was performed to eliminate contamination by parental strains. The resultant diploids were patched onto sporulation medium and the tetrads dissected by micromanipulation. To evaluate the ability of the *S. cerevisiae* clinical isolates to maintain and transmit the [*PSI*⁺] determinant, the [*rho*⁻] derivatives of the *S. cerevisiae* clinical isolates were crossed with 74-D690 [*PSI*⁺]. Resulting [*PSI*⁺] *ade1-14* strains would be expected to give white colonies in complete medium (YEPD) while the [*psi*⁻] strains would give red colonies (Derkatch *et al.*, 1996). Thus the phenotype of the haploid spores from a cross between a [*PSI*⁺] *ade1-14* strain and a [*rho*⁻] Ade⁺ haploid derived from one of clinical isolates was used to determine whether or not the clinical strain could support the [*PSI*⁺] determinant. Similarly, a cross with a [*psi*⁻] derivative of 74-D690 was used to indicate if the clinical isolate carried the [*PSI*⁺] determinant.

Recombinant DNA techniques

Standard protocols were used for DNA isolation, electrophoresis, DNA fragment purification, restriction enzyme digestion and PCR (Sambrook *et al.*, 1989). Restriction enzymes and DNA polymerase (ExpandTM) were purchased from Boehringer Mannheim, 'High Fidelity' *Pwo* polymerase from Roche. Oligonucleotides were purchased from MWG Biotech UK Ltd.

Sedimentation analysis of Sup35p and Rnq1p

Total protein extracts were prepared from various *S. cerevisiae* strains and fractionated into soluble and insoluble (pellet) fractions by centrifugation essentially as described by

Eaglestone *et al.* (1999) for Sup35p and as described by Sondheimer and Lindquist (2000) for Rnq1p. The resulting protein samples were analysed on 10% SDS-polyacrylamide gels and electrophoretically transferred to nitrocellulose (Sartorius) for Western blot analysis, employing an affinity-purified polyclonal antibody raised against recombinant *S. cerevisiae* Sup35p or Rnq1p, respectively, essentially as described by Stansfield *et al.* (1995). Anti-rabbit secondary antibody and the ECL reagent (Pharmacia-Amersham) were used to detect bound antibody according to the manufacturer's protocols. Molecular weights of proteins were estimated using pre-stained molecular weight standard markers (Sigma).

PCR amplification and sequencing of various prion-forming domains

The PrD M-encoding region of the *SUP35* gene of *S. cerevisiae* was amplified from the *S. cerevisiae* clinical isolates using the 'High Fidelity' *Pwo* polymerase (Roche) which contains a 3'-5' proofreading activity, together with the oligonucleotide primers: SUP35-FOR and P3 with extensions bearing *Bam*HI and *Xho*I restriction sites respectively (Table 3). The resulting PCR products were directly sequenced, by the chain termination method (Sanger *et al.*, 1977), on both strands for each of the 16 *SUP35* PrD M domains with exception of strains SCI1 and SCI5 for which only the PrD-encoding region was sequenced. The sequence data have been deposited with GenBank (Accession no. AY028644 – AY028659; Table 1). Sequencing of the full-length *SUP35* gene from *S. cerevisiae* strains SCI2, SCI3, SCI4 and SCI6 was done after PCR amplification with the primers SUP35-FOR and REV-Xba (Table 3) with the resulting PCR products being sequenced on both strands. Three additional primer pairs were designed (SUP35-1F/1R, SUP35-2F/2R and SUP35-3F/3R; Table 3) to create DNA

Table 3. PCR primers used in this study.

| Primer no. | Oligonucleotide sequence 5'–3' |
|------------------------|--|
| Sup35-For ^a | GTAACAAAAAGGATCCCTCTTCATCGACTTGCTCG |
| P3 ^a | GATGCACTCGAGATCGTTAAACAACCTCGTCATCC |
| REV-Xba ^a | GGGGGGTCTAGAGATGATGCCGAGGGAAGCAGCGAAGG |
| Rev-7 ^a | ACCAGCTTGATATCCTTGCA |
| Sup35-1f | TCTTCATCGACTTGCTGCG |
| Sup35-1r | GTAGATTACCGGCATCAACATG |
| Sup35-2f | CCAGTGCTGATGCCCTTGATC |
| Sup35-2r | GTTAGGCATCAGTAGGGTG |
| Sup35-3f | TATTGCCGCTAAGATGAAGG |
| Sup35-3r | CATTCTGAAATAACGCCGGG |
| Ure2-f | GCTGCAAATTAACCTTGACA |
| Ure2-r | CTCCACGTGACTCATATC |
| New1-f | TACACGACAATCAGTGTC |
| New1-r | CTTCAATGATTAGTTTGATTT |
| Rnq1-f | CTGGCTGCCTTGGCTTCT |
| Rnq1-mr | GATTGAGTTTGTCCACCAC |
| Rnq1-mf | CCTCATTGGCCTCCATG |
| Rnq1-r | GGATGAAAGGCGAACTGA |

a. The SUP35-FOR, P3, REV-Xba and REV-7 oligonucleotides have extensions bearing *Bam*HI, *Xho*I, *Xba*I and *Eco*RV restriction sites (bold/underlined), respectively, and were used to generate DNA fragments for cloning. The remaining primers were for sequencing only.

fragments with overlapping regions to facilitate the sequencing of the full-length *SUP35* gene.

The PrD-encoding regions of the *URE2* (aa 1–97) and *NEW1* (aa 1–153) genes were PCR amplified by PCR using primer pairs URE2-F/URE2-R and NEW1-F/NEW1-R respectively (Table 3). The larger C-terminal PrD-encoding region of the *RNQ1* gene (aa 141–405) was amplified by PCR using primer pairs RNQ1-F/RNQ1-MR and RNQ1-MF/RNQ1-R (Table 3). Resulting PCR products were sequenced, by the chain termination method, on both strands. The sequence data have been deposited with the GenBank database as follows: *RNQ1* alleles (Accession Nos. AY028674–AY028685), *URE2* alleles (Accession nos. AY028692–AY028697) and *NEW1* alleles (Accession Nos. AY028686–AY028691).

Because preliminary sequence data for *RNQ1* indicated heterozygosity at this locus in the strains SCI7, SCI9 and SCI11, two of these strains, SCI7 and SCI11 were sporulated and haploid progeny (SCI7.2/a, SCI7.2/b, SCI7.2/d and SCI11.5/a, SCI11.5/b, SCI11.5/c, SCI11.5/d respectively) was generated. SCI9 was asporogenous and so the two different *RNQ1* alleles were cloned from the diploid strain into pGEM-T Easy (Promega) after PCR amplification and sequenced as described above.

DNA and protein sequence analysis

To identify tandemly repeated DNA sequences in the various sequenced *SUP35*, *URE2*, *NEW1* and *RNQ1* genes of *S. cerevisiae*, the 'Tandem repeats finder' programme (Benson, 1999) was used. Alignment of the nucleotide and protein sequences was carried out using CLUSTALW (Thompson *et al.*, 1994).

Plasmid construction

The construction of the plasmid pUKC1512, carrying the *SUP35* promoter (–919 to –49 with respect to the translation start codon) and the MC-domains of the *SUP35* gene, has been previously described (Resende *et al.*, 2002). Plasmid pUKC1512-SCI3 and pUKC1512-SCI4 were constructed as follows: the PrD-encoding regions of the *SUP35* gene from the *S. cerevisiae* strains SCI3 and SCI4, respectively, were PCR amplified from genomic DNA as a *Bam*HI/*Eco*RV fragment, using primers SUP35-FOR and REV-7 (Table 3). The resulting DNA fragment was then inserted into plasmid pUKC1512 in frame with MC domains of the *ScSUP35* gene.

Plasmid shuffling assay

Wild-type and recombinant *SUP35* genes were transformed into the [*PSI*⁺] and [*psi*⁻] derivatives of the *S. cerevisiae* haploid strain MT700/9d (Resende *et al.*, 2002) that carries the *sup35::kanMX4* allele, and the centromeric plasmid pYK810 (Kikuchi *et al.*, 1988) which carries the *SUP35* and *URA3* genes. These strains were transformed with the various plasmids each carrying the *HIS3* gene and the different *SUP35* constructs. His⁺ transformants were selected in SD-His medium, and single transformants were then streaked onto both SD-His medium and YEPD medium containing 5-

fluoroorotic acid (5-FOA, 1 mg ml⁻¹), to select for Ura⁻ strains that have lost the *URA3*-containing plasmid (Kaiser *et al.*, 1994). From SD-His and 5-FOA plates, individual colonies were isolated and replica plated onto 1/4YEPD, SD-His, SD-Ura, SD-Ade, YEPD + 200 µg ml⁻¹ Geneticin (G418) and YEPD + 3 mM GdnHCl to verify their genotypes and associated phenotypes.

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