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Studies of Sup35p

A Yeast Prion Protein

By

Simon Spencer Eaglestone

A thesis submitted to the University of Kent for the degree of PhD in Biochemistry in the Faculty of Natural Sciences

Department of Biosciences, May 1999

F164835

DECLARATION

No part of this thesis has been submitted in support of any other application for a degree or qualification of the University of Kent or any other University or Institute of learning.

Simon Eaglestone
May 1999

This thesis is composed of five chapters. Whilst the Introduction and Discussion (Chapters 1 & 5 respectively) are written in a standard format, Chapters 2, 3 & 4 have been written in the format of a scientific publication. I declare that I performed all of the experimental work described in this thesis, except for the mathematical expression of a segregational model (see Chapter 3) which was developed by Dr L.W. Ruddock, University of Kent.

This study has generated the following publications:

Eaglestone S., Cox B.S. & Tuite M.F. (1999) Translation termination efficiency can be regulated in *Saccharomyces cerevisiae* by environmental stress through a prion-mediated mechanism. *EMBO J.* **18**: *1974-1981.*

Eaglestone S., Ruddock L.W., Cox B.S. & Tuite M.F. (1999) Guanidine hydrochloride blocks a critical step in the propagation of the prion-like determinant *[PSI$^+$]* of *Saccharomyces cerevisiae. Submitted to Proc. Natl. Acad. Sci. USA.*

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For my daughter, I wish to document that she was the experiment that gave me the most sleepless nights! Despite all that I see and hear through my work, nothing can beat her when it comes to making me stop and look at the world we live in.

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Abbreviations used in this study

ADE2	The *ADE2* gene encodes the Ade2p protein, which catalyses a reaction in the biosynthetic pathway of adenine.
ade2-1	A mutant allele of the *ADE2* gene that bears a premature nonsense (STOP) codon and therefore produces a non-functional Ade2p protein.
Ade2p	Protein product of the *ADE2* gene that catalyses a reaction in the biosynthesis of adenine.
ATCase	Aspartate transcarbamylase. ATCase catalyses the formation of USA from carbamyl phosphate and aspartate. ATCase mutants are employed in the study of *[URE3]* to monitor USA uptake.
ATPase	An ATP-hydrolysing protein.
CD	Circular dichroism (i.e. CD spectrometry).
CJD	Creutzfeldt-Jakob disease; a human prion disease.
fCJD	Familial CJD; an inherited human prion disease.
sCJD	Sporadic CJD; a 'spontaneous' human prion disease.
DNA	Deoxyribonucleic acid.
EF-1α	Elongation factor 1α.
eRF	Eukaryote polypeptide release factor.
eRF1	Eukaryote release factor 1, sub-unit of the translation-termination factor eRF. eRF1 is Sup45p, the protein product of the *SUP45* gene.
eRF3	Eukaryote release factor 3, sub-unit of the translation- termination factor eRF. eRF3 is Sup35p, the protein product of the *SUP35* gene.
et al.	*Et alia* (and others).
EtOH	Ethanol.
FFI	Fatal familial insomnia; an inherited human prion disease.
FTIR	Fourier transform infrared (i.e. FTIR spectroscopy).
Gdm^+	Guanidinium; the protonated cation of guanidine.
GFP	Green fluorescent protein.
GSS	Gerstmann-Straüssler-Scheinker syndrome; a human prion disease.
GuHCl	Guanidine hydrochloride.
hrs	Hours.

HSP104	The *HSP104* gene encodes the Hsp104p protein.
Hsp104p	The yeast heat shock protein 104 confers tolerance to many forms of stress and is essential for the maintenance of *[PSI⁺]*.
HuPrP	Human PrP.
LD_{50}	Lethal dose 50% (e.g. exposure to lethal temperature that elicits 50% viability).
mins	Minutes.
ml	Millilitre (10^{-3} litre).
μl	Microlitre (10^{-6} litre).
M	Molarity (moles per litre).
mM	Millimolar (10^{-3} moles per litre).
mRNA	Messenger ribonucleic acid.
MoPrP	Mouse PrP.
nm	Nanometre (10^{-9} metre).
OD_{600}	Optical (culture) density measured at 600nm.
pers. comm.	Personal communication.
PFD	Prion forming domain.
PNM	*Psi No More* – loss of the *[PSI⁺]* phenotype.
PNM?	A putative arginine-modifying enzyme whose function is required for the maintenance of *[PSI⁺]*.
Prnp	The *Prnp* gene encodes PrP.
PrP	Mammalian prion protein.
PrP*	A partially unfolded conformer of PrP.
PrP^{c}	Native (cellular) conformer of PrP.
PrP^{Sc}	Aberrant (scrapie) disease-associated conformer of PrP.
PrP27-30	Protease-resistant core derived from PrP.
[PSI]	A yeast prion allosuppressor determinant; *[PSI]* status reflects the conformational properties of Sup35p.
[PSI⁺]	'Transfected' state/phenotype (i.e. in the presence of the *[PSI]* determinant = Sup35p prion conformer).
[psi⁻]	*[PSI]*-free state/phenotype. (i.e. in the absence of the *[PSI]* determinant = Sup35p prion conformer is absent).

SAF	Scrapie-associated fibrils; highly ordered disease-associated polymeric structures of PrP^{Sc}.
Sal^+	Wild type non-suppressed phenotype (i.e. translation termination efficient).
SAL3	The *SAL3* locus is the *SUP35* gene that encodes Sup35p = eRF3.
sal3	A recessive allosuppressor mutant of the *SAL3* = *SUP35* gene.
SDS	Sodium dodecyl sulfate.
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis.
SUP35	The *SUP35* gene encodes the Sup35p protein.
Sup35p	Prion protein product of the *SUP35* gene, whose properties manifest as the *[PSI^+]* phenotype. Sup35p is also eRF3, a sub-unit of the eukaryotic translation-termination factor eRF.
SUQ5	*SUQ5* is a weak seryl-inserting ochre (UAA) nonsense suppressor tRNA.
tRNA	Transfer ribonucleic acid.
[URE3]	'Transfected' state/phenotype (i.e. in the presence of the *[URE3]* determinant = Ure2p prion conformer).
[ure3]	*[URE3]*-free state/phenotype. (i.e. in the absence of the *[URE3]* determinant = Ure2p prion conformer).
URE2	The *URE2* gene encodes the Ure2p protein.
ure2	Non-functional mutants of the *URE2* gene.
Ure2p	Prion protein product of the *URE2* gene, whose properties manifest as the *[URE3]* phenotype. Ure2p is a regulatory protein that represses nitrogen catabolism genes.
USA	Ureido succinic acid; a metabolite employed in the identification of the *[URE3]* = *ure2* phenotype.
YEPD	Standard rich complete media used for the growth of yeast.
¼YEPD	Modified YEPD for enhancement of yeast colony colour.

ABSTRACT

[PSI⁺] is a protein-based heritable phenotype of the yeast *Saccharomyces cerevisiae* that reflects the prion-like properties of the chromosome-encoded protein Sup35p. This protein is known to be an essential eukaryote polypeptide release factor, namely eRF3. In a *[PSI⁺]* strain, the prion conformer of Sup35p exists predominantly as large oligomers, which results in the intracellular depletion of functional release factor (i.e. eRF3) and hence inefficient translation termination. Intriguingly, the prion conformer of Sup35p can be eliminated from *[PSI⁺]* strains by growth in the presence of the protein denaturant guanidine hydrochloride (GuHCl). Strains are 'cured' of *[PSI⁺]* by millimolar concentrations of GuHCl, well below that normally required for protein denaturation. It was shown that the kinetics of GuHCl-induced curing fit a segregational model, whereby the heritable *[PSI⁺]* determinant is diluted from a culture following the total inhibition of prion replication. A hypothesis for the mechanism of curing is proposed namely that the guanidinium cation inhibits an arginine-modifying enzyme, whose action is required for the post-translational modification of Sup35p and ultimately *[PSI⁺]* maintenance. The *[PSI⁺]* determinant does not elicit a disease state in yeast, rather it was shown to confer a selective phenotypic advantage namely enhanced stress tolerance. Moreover, it was demonstrated that the efficiency of translation termination is regulated by environmental stress through a prion-mediated mechanism. This study has addressed the relationship between Sup35p, *[PSI⁺]* and stress proteins of *S.cerevisiae* and revealed that prion proteins are not simply pathogenic misshapen proteins and that they may serve as a novel means to regulate many cellular processes in fungi.

Chapter I

Introduction

Chapter

I

Introduction

1.1 From genes to proteins, with something in-between

Half a century ago, the work of Beadle and Tatum began to unravel the biochemical basis of genes and inheritance and gave rise to the 'one gene – one enzyme' hypothesis (Beadle, 1945). Today, this hypothesis still remains partially valid, although it does require some modification. For example, it is now known that many genes encode proteins that are not enzymes or polypeptides that serve as subunits within multimeric protein complexes. Other genes encode ribosomal and transfer ribonucleic acids, which fulfil structural and functional roles respectively. In 1944, Avery, MacLeod and McCarty first demonstrated that deoxyribonucleic acid (DNA) was capable of conveying genetic information, in experiments employing the *Pneumococcus* bacterium (Avery *et al.*, 1944). Ironically, at that time the accepted 'vehicle' of inheritance was protein and their observations were largely ignored. Yet less than a decade later, Hershey and Chase were also able to conclude that DNA was able to transfer genetic information and that DNA was in fact the sole factor, by which the bacteriophage T2 was able to transfect *Escherichia coli* (Hershey & Chase, 1952).

It was the landmark scientific achievement of Watson and Crick that finally established DNA as the molecule of inheritance (Watson & Crick, 1953a, b). Crick summarised the relationship between DNA, RNA and protein in his 'Central dogma of molecular biology'; DNA directs its own replication and its transcription to RNA that, in turn, directs its translation to protein (Crick, 1970). Likewise, RNA can direct its own replication and the production of DNA and protein. However, the dogma states that proteins can only be the recipients of genetic information and that protein cannot specify DNA, RNA or protein. 'Once [sequential] information has passed into a protein, it cannot get out again' (Crick, 1958).

Whilst genetic information defines the chemical properties of a protein (i.e. its primary sequence), biological activity is a consequence of a second component to the genetic code, namely protein folding. As stated by Anfinsen (1973), the 'biological function [of

proteins] appears to be more a correlate of macromolecular geometry than of chemical detail'. Furthermore, 'no special genetic information, beyond that contained in the amino acid sequence, is required for the proper folding of the molecule and for the formation of correct disulfide bonds' (Goldberger *et al.*, 1963). The folding of a polypeptide can also be governed by the temperature, pH and ionic strength of the aqueous environment (Anfinsen, 1973; Kelly, 1996) and by other ancillary factors, such as stress proteins or molecular chaperones (reviewed in Ellis, 1998; Netzer & Hartl, 1998). The ability of certain proteins to adopt more than one conformation can produce several protein conformers, of varying structure and activity, from the one gene product. Such variation can have catastrophic consequences, as is the case with the family of amyloidogenic proteins, which are responsible for many human diseases, including Alzheimer's disease and Creutzfeldt-Jakob disease (Kelly, 1996; Wetzel, 1996; Carrell & Lomas, 1997). In the last decade, it has become apparent that the sole agent responsible for a family of transmissible neurodegenerative diseases, might actually be an aberrant self-replicating protein conformer, namely a 'prion' (Griffith, 1967; Prusiner, 1982; Horwich & Weissman, 1997; Prusiner *et al.*, 1998).

Incredibly, there are now three examples of prion-based inheritance in fungi (Wickner, 1994; Tuite & Lindquist, 1996; Coustou *et al.*, 1997). In each of these cases, a single host-encoded polypeptide is able to adopt two (or more) conformations. The wild type and 'aberrant' conformers exhibit different biological activities and hence, give rise to different phenotypic properties. The prion conformer is able to mediate its own replication, by promoting the structural rearrangement of the wild type protein to the same aberrant conformation. Rather than confer disease, the self-propagation of the prion defines its behaviour as a dominant, heritable determinant. Whilst proteins are indeed the recipients of genetic information, a number do possess the ability to convey genetic (i.e. phenotypic) information, encrypted within their three dimensional structure. Since 1953 and the revelation of the structure of DNA, scientists have studied every aspect of the genetic code at the molecular level. Even more recently, it has become possible to study the behaviour of polypeptides in solution and to begin to decipher the second half of the genetic code, namely protein folding. Yet, despite all that has been learnt, it is ironic that the prion has prompted the study of a near-forgotten notion, namely that of protein-based inheritance.

1.2 Genetics of *[PSI⁺]*

1.2.1 Translation termination, nonsense suppression and *[PSI⁺]*

The yeast *Saccharomyces cerevisiae* has been essential in the elucidation of the mechanisms by which genes are translated into proteins. Translation encompasses three stages, namely initiation, elongation and termination. Each stage employs several protein factors, which mediate the decoding of genetic information and ensure the fidelity of translation. Initiation comprises the assembly of the ribosome and its adopting a state of readiness to decode the messenger RNA (mRNA). Translation proceeds via a repetitive cycle of elongation, whereby each encoded amino acid is delivered to the translating ribosome by a transfer RNA (tRNA) and attached to the growing polypeptide chain. Finally, when the nascent polypeptide is complete, the process is terminated and the protein is released from the ribosome. By studying mutations that confer deficiencies of translation to yeast, the eukaryotic translation apparatus has been dissected and characterised. It was during the study of eukaryote translation termination, that Cox (1965) discovered a heritable 'mutation', that he termed *[PSI⁺]*. This dominant phenotypic element did not appear to be associated with any nucleic acid, but nonetheless, was able to exert a marked effect upon the efficiency of translation termination (Young & Cox, 1972; Tuite *et al.*, 1982; Cox *et al.*, 1988).

The unravelling of translation termination in yeast and in particular, the identity of the eukaryotic polypeptide release factors began with the study of 'supersuppressors' and mutations that induced errors in the fidelity of translation termination (Hawthorne & Mortimer, 1963; Cox, 1965). Super-suppression was defined as "a state in which the phenotypes of specific alleles, at a variety of loci, are simultaneously reversed to a pseudo-wild type condition by the presence of a mutation at an external locus" (Young & Cox, 1971). Supersuppressors encompass mutant tRNA genes, which encode tRNAs that bear anticodons which recognise termination signals (Goodman *et al.*, 1977; Broach *et al.*, 1981; Waldron *et al.*,1981). Such mutant tRNA's are able to 'translate' nonsense codons and insert an amino acid, thereby facilitating further polypeptide growth rather

than polypeptide chain termination. Such 'read-through' of nonsense codons is termed nonsense suppression. The nonsense suppressor tRNA's are classified by their codon specificity, decoding either ochre (UAA), amber (UAG) or opal (UGA) termination signals. There exists a second class of nonsense suppressor that does not exhibit codon-specificity and is therefore unlikely to encode mutant tRNA (Table 1.1). So-called 'omnipotent' nonsense suppressor mutations cause suppression of all three classes of termination codon. The omnipotent suppressor mutations provide a means by which the components of the eukaryote translation termination apparatus can be identified (Hawthorne & Leupold, 1974; Ono *et al.*, 1979; Crouzet & Tuite, 1987; Crouzet *et al.*, 1988). Finally, there are mutations or elements which themselves influence the activity of nonsense suppressors (Sherman, 1982). These 'suppression modifiers' may either enhance or decrease the efficiency of nonsense suppression and are termed allosuppressors (Hawthorne & Leupold 1974; Cox, 1977) and antisuppressors respectively (McCready & Cox, 1973; Laten *et al.*, 1978).

Table 1.1 **Classification of nonsense suppressor mutations in *S.cerevisiae*.**

Class	Properties	Identity
Nonsense suppressors	Strong codon specificity: (UAA) Ochre (UAG) Amber (UGA) Opal	Mutant tRNA
Omnipotent suppressors	Non codon-specific	Mutant protein
Allosuppressors	Enhance nonsense suppression	Mutant protein
Antisuppressors	Decrease nonsense suppression	Mutant protein

The study of nonsense suppression has been facilitated by the use of a sensitive genetic marker, the *ade2-1* allele (Cox, 1965). The *ade2-1* allele bears an internal nonsense mutation and fails to encode a functional Ade2p polypeptide product, which catalyses a

step in the biosynthesis of adenine, thereby conferring an adenine-dependence (*Ade⁻*) to an *ade2-1* strain (Table 1.2). Furthermore, when grown under adenine-limited conditions, the adenine auxotroph accumulates a red pigment (Cox, 1965; Chaudhuri *et al.*, 1996) which forms the basis of a simple colony-colour phenotypic screen. By screening for growth on complete synthetic media lacking adenine or for the appearance of white colonies, the *ade2-1* marker is a valuable tool for the isolation of both omnipotent (i.e. non-tRNA) suppressor mutations and allosuppressors. *SAL3* was one such allosuppressor locus (Cox, 1977). Recessive *sal3* mutants were identified using the *ade2-1* marker, by virtue of its ability to enhance the activity of a weak nonsense suppressor tRNA, namely *SUQ5*. Intriguingly, Cox had previously discovered another allosuppressor determinant, *[PSI⁺]* (Cox, 1965). Whilst *sal3* mutants and *[PSI⁺]* strains exhibited a similar translation termination deficiency, the two determinants exhibited markedly different genetics (Cox, 1977; see Chapter 1.2.2).

Genotype		Colony colour	Auxotrophy
ADE2	ATG ——— STOP TAA	White	*Ade⁺*
ade2-1	ATG ——— STOP TAA ——— STOP TAA	Red	*Ade⁻*
ade2-1	*SUQ5, [psi⁻]*	Red	*Ade⁻*
ade2-1	*SUQ5, [PSI⁺]*	White	*Ade⁺*
ade2-1	*SUQ5, [psi⁻], sal3*	White	*Ade⁺*

Table 1.2 The *ade2-1* allele as a reporter of nonsense suppression in yeast.

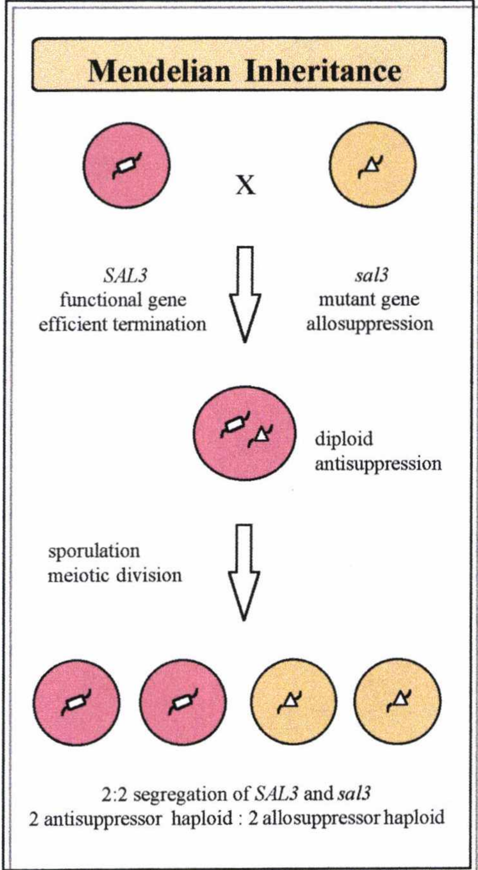
The *ade2-1* marker is a nonsense (ochre) mutant allele of the *ADE2* gene. The full-length *ADE2* gene product functions in adenine biosynthesis and catalyses the metabolism of a red pigment. The *ade2-1* allele can be used to monitor the level of nonsense suppression, in strains bearing nonsense suppressors, such as *SUQ5;* a weak seryl-inserting ochre tRNA (Waldron *et al.*, 1981). Suppression levels can be determined on the basis of colony colour and/or by growth on complete synthetic media lacking adenine.

Like the *sal3* mutation, *[PSI⁺]* was originally identified as a modifier of translation suppression, that enhances the activity nonsense suppressor tRNA [e.g. *SUQ5*] (Cox, 1965, 1977). *SUQ5*, also designated *SUP16,* is a seryl-inserting nonsense-suppressor tRNA (Ono *et al.*, 1979; Waldron *et al.*, 1981). *SUQ5* is a weak ochre (UAA) suppressor and alone cannot suppress the *ade2-1* mutation, such that a yeast strain bearing both the *ade2-1* and *SUQ5* markers grows to form red adenine-requiring colonies (Table 1.2). By contrast, the allosuppressor properties of a *sal3* mutation enhance the activity of the suppressor *SUQ5* and permit adenine-independent growth of white colonies. Whilst many strong ochre suppressors elicit dominant suppression in a *[psi⁻]* background, most are lethal or extremely toxic in conjunction with the *[PSI⁺]* determinant (Cox, 1965, 1977). Employing *ade2-1* strains, *[PSI⁺]* has also been shown to enhance the activity of opal (UGA) suppressors (Schmitt & Olson, pers. comm. cited in Cox *et al.*, 1988) but not that of amber (UAG) suppressors (Cox *et al.*, 1988). However, using an *in vitro* assay system, it is demonstrable that *[PSI⁺]* enhances the efficiency of all three classes (ochre, opal and amber) of nonsense suppressor tRNA (Tuite *et al.*, 1983). Furthermore, the presence of the *[PSI⁺]* element also increases the level of suppression induced by frameshift suppressors (Culbertson *et al.*, 1977; Cummins *et al.*, 1980) and by the aminoglycoside antibiotic paromomycin (Palmer *et al.*, 1979a, b). *[PSI⁺]* is an allosuppressor, in that it acts only by modifying the activity of endogenous factors, which themselves possess the ability to influence the fidelity of translation (Mundy, 1979; Cox *et al.*, 1988).

1.2.2 Inheritance of *[PSI⁺]* and other allosuppressor determinants

The *[PSI⁺]* and *sal3* allosuppressor determinants were both identified as modifiers of nonsense suppression in yeast, by virtue of screens which utilised the *ade2-1 / SUQ5* marker system (Cox, 1965, 1977). The same markers also permit a genetic analysis of these 'mutations'. When a haploid strain bearing the *sal3* determinant is crossed with a *sal⁺* tester strain, the resulting diploid exhibits an antisuppressor phenotype, i.e. the activity of the nonsense suppressor tRNA is repressed and the diploid grows on adenine-containing media, producing red colonies (Cox, 1977). Thus, the *sal3* allosuppressor determinant is recessive. Upon sporulation (i.e. meiotic division), the diploid tetrads give

rise to 2 suppressed to 2 non-suppressed haploid progeny. The *sal3* genetic determinant thus behaves as a recessive mutant allele of the *SAL3* locus and exhibits a classic pattern of Mendelian inheritance (Figure 1.1). Intriguingly, the *[PSI⁺]* determinant exhibits completely different genetic properties. First, *[PSI⁺]* is a dominant phenotype since the mating of a *[PSI⁺]* haploid with a *[psi⁻]* haploid generates a diploid, that exhibits an allosuppressor phenotype (Cox, 1965). Second, as illustrated in Figure 1.2, *[PSI⁺]* exhibits a non-Mendelian pattern of inheritance, whereby sporulation of a diploid produced by a *[PSI⁺]* x *[psi⁻]* mating, only yields [*PSI⁺*] haploid spores (Cox, 1965; Cox *et al.*, 1988).



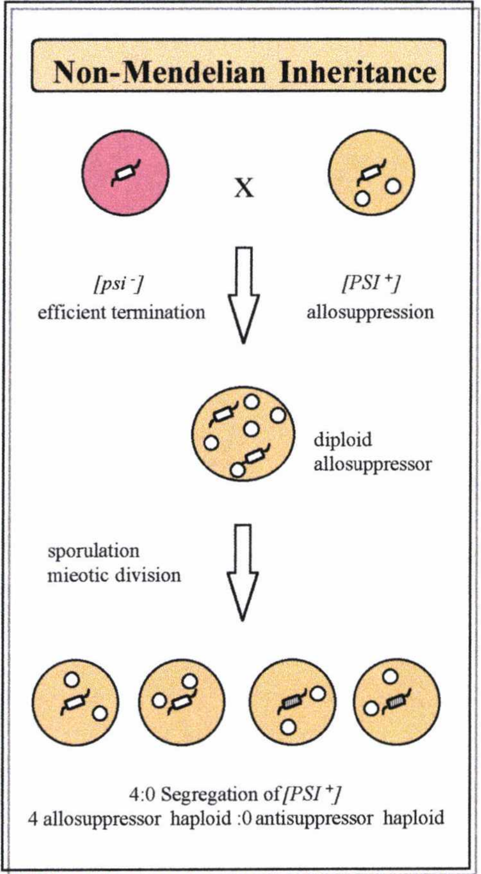


Figure 1.1 The *ade2-1 / SUQ5* markers permit the assessment of the genetic properties of allosuppressor determinants in yeast (Cox, 1965, 1977). A haploid *sal⁺* tester strain (bearing *ade2-1 / SUQ5* markers) is crossed with a strain bearing an allosuppressor determinant (as well as the *ade2-1 / SUQ5* mutations). The colour and adenine-requirements of the resulting diploid, demonstrate whether the allosuppressor is a recessive or a dominant determinant. The diploid can then be induced to sporulate and undergo a meiotic cycle of cell division. The properties of the resulting haploid progeny demonstrate whether the determinant is a nuclear mutation (△) or a cytoplasmic extrachromosomal element (O).

The abnormal pattern of inheritance is best explained, as the dominant phenotype *[PSI⁺]* is the consequence of a cytoplasmic determinant (Cox, 1965; Young & Cox, 1971). Indeed, it is demonstrable that the dominant *[PSI⁺]* factor can be transfected to *[psi⁻]* strains by cytoduction (Tuite, 1978), a form of mating in which cytoplasmic contents are exchanged but nuclear fusion is precluded (Conde & Fink, 1976). Several extrachromosomal heritable elements have been described in yeast. These include mitochondrial DNA (Ephrussi *et al.*, 1954), the killer character (Somers & Bevan, 1969; Berry & Bevan, 1972), the *[URE3]* mutation (Lacroute, 1971), the 2μm plasmid (Livingston, 1977) and numerous RNA-based virus-like elements (reviewed in Wickner *et al.*, 1996). To date, *[PSI⁺]* has demonstrated no association with these or any other extrachromosomal nucleic acid species (Young & Cox, 1972; Tuite *et al.*, 1982).

1.2.3 Elimination of the *[PSI⁺]* phenotype

The cytoplasmic determinant *[PSI⁺]* can be eliminated from yeast both by nuclear mutation and by growth in the presence of certain chemical agents. Tuite *et al.* (1981a) observed that *[PSI⁺]* strains could be liberated, or 'cured', of the extrachromosomal *[PSI⁺]* element by growth in the presence of millimolar concentrations of guanidine hydrochloride (GuHCl). Whilst this reagent is not a mutagen, it is known to elicit the loss of the penicillinase-encoding plasmid of *Staphylococcus aureus* (Juliani *et al.*, 1975) and it produces non-chromosomal respiratory-deficient petites of *S.cerevisiae* (Juliani *et al.*, 1973). Furthermore, the frequency of *[PSI⁺]* to *[psi⁻]* conversion is elevated by growth in the presence of alcohols, dimethylsulfoxide (Tuite *et al.*, 1981a) and in hypertonic media (Singh *et al.*, 1979). The mechanism by which these reagents elicit the loss of the *[PSI⁺]* determinant is unknown.

Young and Cox (1971) described the first so-called *Psi No More (PNM)* mutation (McCready *et al.*, 1977). When a *[PSI⁺]* strain was crossed with a strain bearing a *PNM* mutation (formerly called *R*), the *[PSI⁺]* determinant was eliminated (Young & Cox, 1971) with the diploid and the majority of haploid spores, arising from the diploid, being *[psi⁻]*. Young and Cox proposed two explanations: (1) a wild-type copy of the *PNM* gene was required for *[PSI⁺]* maintenance, or alternatively (2), the dominant nuclear *PNM*

mutation might have prevented the replication of the extrachromosomal *[PSI⁺]* determinant. To date, two *PNM* genes have been identified and characterised with respect to their role in *[PSI⁺]* maintenance. Doel *et al.* (1994) first cloned and sequenced a *PNM* gene, namely *SUP35*. The following year, a second *PNM* gene was identified as being *HSP104* (Chernoff *et al.*, 1995a). By definition the 'wild-type' products of these genes are essential for the *[PSI⁺]* determinant and its associated phenotype. The properties of the *SUP35* gene product and its interaction with the stress protein Hsp104p, ensure the maintenance and propagation of the *[PSI⁺]* determinant (see Chapter 1.4.3).

1.3 Yeast prions

1.3.1 The identity of the *[PSI⁺]* determinant

To facilitate the biochemical analysis of the yeast allosuppressor mutants, Tuite *et al.* (1981b) developed a homologous *in vitro* assay for nonsense suppression. Surprisingly, whilst *[PSI⁺]* is dominant to *[psi⁻]* *in vivo* (Cox, 1965), the reverse is true *in vitro* (Tuite *et al.*, 1983). A mixture of *[PSI⁺]* and *[psi⁻]* cell-free lysates did not support read-through of nonsense codons *in vitro*. Lysates prepared from a *[psi⁻]* strain appeared to possess a ribosome-associated factor, that could inhibit the action of a nonsense suppressor tRNA and promote efficient translation termination (Tuite *et al.*, 1987). Furthermore, this factor appeared to be a protein, since it was readily washed from ribosomes using high salt buffers and was inactivated by heat. It was proposed that this inhibitory factor was the eukaryote polypeptide release factor (eRF) and that *[PSI⁺]* strains are defective for translation termination, since they produce defective RF or reduced levels of the normal eRF protein (Tuite *et al.*, 1987).

Ultimately, the identity of this putative RF was revealed following the isolation and characterisation of a particular omnipotent suppressor locus, namely *SUP35*. The *SUP35* allosuppressor locus (Hawthorne & Leupold, 1974) has been identified by many investigators and has also been called *sal3* (Cox, 1977), *SUP2* (Inge-Vechtomov & Andrianova, 1970), *GST1* (Kikuchi *et al.*, 1988) and *suf12* (Wilson & Culbertson, 1988).

Intriguingly, Kushnirov *et al.* (1988) and Wilson and Culbertson (1988) identified the *SUP35* gene product, as a fusion protein derived from an elongation factor. Strains of *E.coli* that bear mutations in the gene encoding the bacterial elongation factor EF-Tu, can exhibit an omnipotent suppressor phenotype (Vijgenboom *et al.*, 1985). Similarly, mutation of the yeast elongation factor homologue EF-1α can also enhance nonsense suppression (Song & Liebman, 1986), but the *SUP35* locus does not map to either the *TEF1* or the *TEF2* genes, which encode EF-1α (Crouzet & Tuite, 1987). Despite the extensive description of mutations at the *SUP35* locus, all of which give rise to the same allosuppressor phenotype as *[PSI$^+$]*, none of them exhibit a non-Mendelian pattern of inheritance (Inge-Vechtomov & Andrianova, 1970; Hawthorne & Leupold, 1974). However, Sup35p was shown to be a eukaryote release factor, eRF3 (Stansfield *et al.*, 1995a; Zhouravleva *et al.*, 1995), thereby clarifying the allosuppressor properties of *sal3* (i.e. *SUP35)* nuclear mutations.

The genetic properties of the *[PSI$^+$]* determinant were less easily explained. Certain nonsense suppressor mutations of the *SUP35* gene were known to be incompatible with *[PSI$^+$]* (Cox, 1977), whilst multicopy plasmids carrying *SUP35*, gave rise to slow growth and were unstable in a *[PSI$^+$]* background (Dagkesamanskaya & Ter-Avanesyan, 1991). The plasmid-mediated overexpression of the entire *SUP35* gene (Chernoff *et al.*, 1988; Kushnirov *et al.*, 1990), or the overexpression of only the 5' region of *SUP35* was shown to elicit an enhancement of nonsense suppression i.e. a defect in translation termination (Chernoff *et al.*, 1992; Ter-Avanesyan *et al.*, 1993). Together, the overexpression of *SUP35* and the *[PSI$^+$]* determinant appeared to preclude translation termination to such a degree, that cells became non-viable. This was supported by the demonstration that the *SUP35* gene and its RF product were essential for viability, regardless of the presence or absence of *[PSI$^+$]* (Wilson & Culbertson, 1988; Dagkesamanskaya & Ter-Avanesyan, 1991).

The break-through that finally led to the identification of the *[PSI$^+$]* determinant was the demonstration that the overexpression of *SUP35* gene in *S.cerevisiae* induced the *de novo* appearance of the *[PSI$^+$]* determinant and its corresponding allosuppressor phenotype (Chernoff *et al.*, 1993). Two independent laboratories then confirmed an association between *SUP35* and *[PSI$^+$]*. Doel *et al.* (1994) cloned and sequenced a *PNM* mutation,

which was shown to encode an amino acid substitution within the N-terminus of the *SUP35* gene product, Sup35p. Deletion analysis of the *SUP35* gene revealed that the N-terminus of Sup35p was non-essential for viability, but essential for the establishment and maintenance of *[PSI⁺]* (Ter-Avanesyan *et al.*, 1994).

The overexpression of the first 114 amino acid residues of Sup35p was shown to be sufficient for the maintenance of the *[PSI⁺]* determinant and for the induction of nonsense suppression (Ter-Avanesyan *et al.*, 1994). Conversely, the expression of a truncated Sup35p, lacking this 'allosuppressor' N-terminal domain, resulted in a dominant antisuppressor phenotype, but was sufficient to maintain viability (Ter-Avanesyan *et al.*, 1994). 'Expression of Sup35p [is] necessary, but not sufficient for the *[PSI⁺]* phenotype, since strains carrying the wild-type *SUP35* gene may either possess or not the *[PSI⁺]* determinant' (Ter-Avanesyan *et al.*, 1994). It was proposed that Sup35p was a 'trans-acting' factor in the maintenance of *[PSI⁺]*; whilst the expression of Sup35p was essential for *[PSI⁺]*, Sup35p was not thought to be the *[PSI⁺]* determinant itself (Doel *et al.*, 1994; Ter-Avanesyan *et al.*, 1994). Clearly, Sup35p interacted with the *[PSI⁺]* determinant (Table 1.3) and it was proposed that in *[PSI⁺]* cells, this affinity 'titrated Sup35p away from termination' giving rise to an allosuppressor phenotype (Doel *et al.*, 1994).

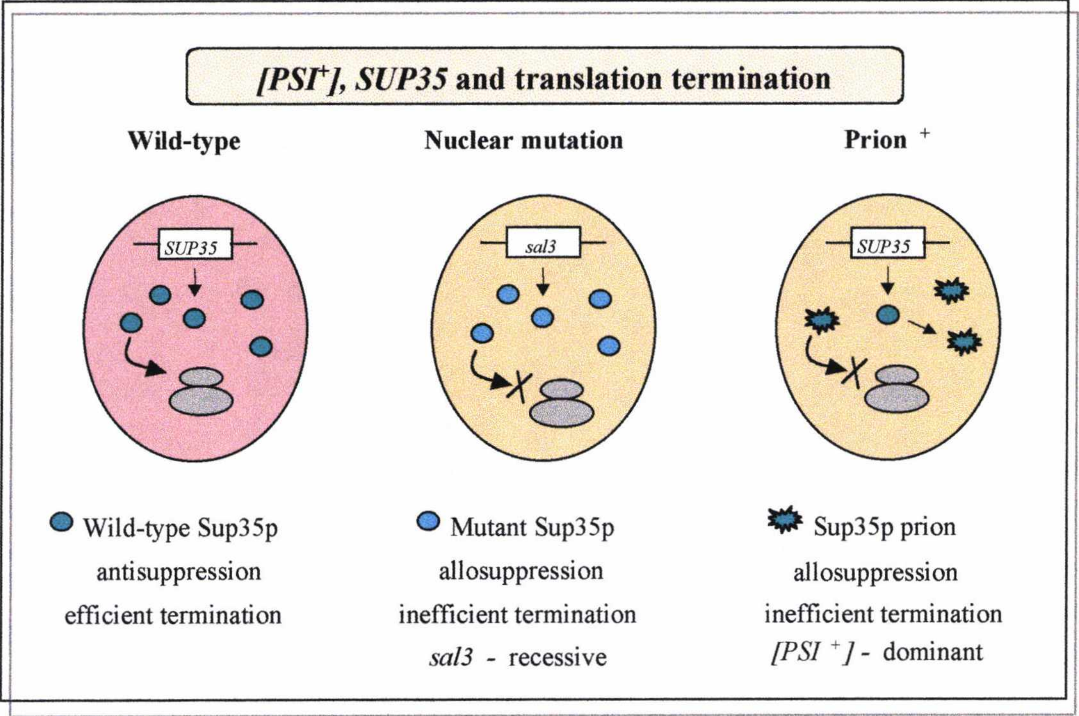
Table 1.3 *SUP35* gene and the *[PSI⁺]* allosuppressor determinant are associated.

Observation	Reported
SUP35 identified as an allosuppressor locus	Hawthorne & Leupold (1974)
[PSI⁺] lysates lack a putative release factor	Tuite *et al.* (1987)
SUP35 encodes an elongation factor-like protein	Kushnirov *et al.* (1988)
	Wilson & Culbertson (1988)
Overexpression of *SUP35* induces *[PSI⁺]*	Chernoff *et al.* (1993)
Psi No More mutations mapped to 5' *SUP35*	Doel *et al.* (1994)
5' deletion of *SUP35* eliminates *[PSI⁺]*	Ter-Avanesyan *et al.* (1994)
Sup35p is eukaryote release factor eRF3	Stansfield *et al.* (1995a)

1.3.2 *[PSI⁺]* and *[URE3]*: yeast prions

Reed Wickner successfully explained the nature of the *[PSI⁺]* determinant, when he made the inspirational connection between two yeast extrachromosomal determinants, *[PSI⁺]* and *[URE3]*, and the transmissible scrapie agent (Wickner, 1994). *[PSI⁺]* and *[URE3]* are proposed to be prions of *S.cerevisiae* (Cox *et al.*, 1994; Wickner, 1994; Wickner *et al.*, 1995; Tuite & Lindquist, 1996). In 1982, Stanley Prusiner introduced the term 'prion' to describe the nature of the infectious agent responsible for scrapie, a neurodegenerative disease of sheep (Prusiner, 1982). 'Prions are elements that impart and propagate variability through multiple conformers of a normal cellular protein' (Prusiner *et al.*, 1998). A prion is a self-replicating protein particle that lacks nucleic acid (reviewed in Prusiner, 1991; Weissmann, 1994a; Prusiner *et al.*, 1998).

Like *[PSI⁺]*, *[URE3]* is a dominant cytoplasmic heritable determinant (Lacroute, 1971; Wickner, 1994). Similarly, *[URE3]* requires expression of the host-encoded *URE2* gene and is incompatible with certain nuclear *ure2* mutations (Lacroute, 1971; Aigle & Lacroute, 1975). The *[URE3]* determinant and *ure2* mutations ablate repression of nitrogen catabolic enzymes, which would otherwise occur upon growth in the presence a good nitrogen source (Lacroute, 1971; Courchesne & Magasanik, 1988; Wickner, 1994). Aspartate transcarbamylase (ATCase) catalyses the production of ureidosuccinate (USA), a biosynthetic precursor of uracil (Lacroute, 1971). Strains lacking ATCase grow on media supplemented with USA, but growth is repressed by the presence of ammonium. However, in the presence of *[URE3]*, such strains take up USA and grow even in the presence of the nitrogen source. Wickner (1994) stated that the *[URE3]* phenotype could reflect a stable conformational change of normal Ure2p, into an inactive prion conformer (Figure 1.2). Whereas ammonium would usually trigger Ure2p to block the uptake of USA, the prion conformer would be unable to perform this function. Similarly, it was proposed that the allosuppressor phenotype *[PSI⁺]* could be the consequence of the prion-like properties of Sup35p. In its aberrant prion-like conformer, Sup35p (i.e. eRF3) would be unable to mediate translation termination, thereby enhancing the activity of nonsense suppressors (Cox, 1994; Wickner, 1994).



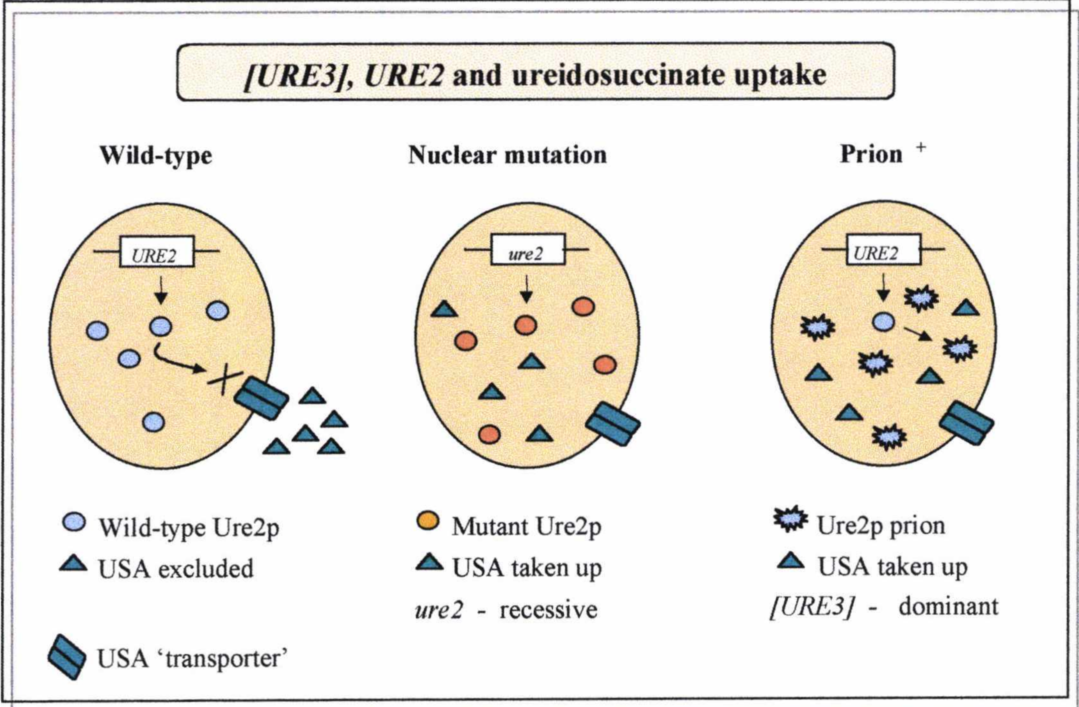


Figure 1.2 Wickner's yeast prion hypothesis. The genetics of *[PSI⁺]* and *[URE3]* reflect the prion-like properties of Sup35p and Ure2p respectively (Wickner, 1994; Weissmann, 1994b). The prion-associated phenotype is the same as that of recessive, loss-of-function mutations: *[PSI⁺]* cells exhibit inefficient translation termination, whereas *[URE3]* strains take up ureidosuccinate, from ammonia-enriched media. Unlike the nuclear mutations, the prion elicits a dominant phenotype, since the prion catalyses the conversion of the normal protein to the same inactive prion conformer.

Several extraordinary genetic properties of the *[URE3]* and *[PSI⁺]* determinants (Table 1.4) are thus readily explained, within the framework of the yeast prion hypothesis. (1) *[URE3]* and *[PSI⁺]* both behave as dominant, non-Mendelian determinants (Cox, 1965; Lacroute, 1971) and can be 'transmitted' by cytoduction (Aigle & Lacroute, 1975; Tuite, 1978). These properties are consistent with the proposed cytoplasmic localisation of a prion and its ability to 'corrupt' wild type protein to the same aberrant conformation. (2) The prion-associated phenotypes can be eliminated, by growth in the presence of 5mM GuHCl (Tuite *et al.*, 1981a; Aigle *cited in* Cox *et al.*, 1988; Wickner, 1994), but the loss of the prion is reversible. Following growth of pure 'cured' colonies, *[PRION⁺]* strains can again be isolated using selective media (Cox *et al.*, 1988; Wickner, 1994; Wickner & Masison, 1996). Reversible curing is not consistent with a nucleic-acid determinant, such as a plasmid. (3) The rate of spontaneous prion appearance is greatly enhanced (~100 fold) by the overexpression of the wild-type gene (Chernoff *et al.*, 1993; Wickner, 1994; Masison & Wickner, 1995). The greater the concentration of protein, the more likely that it would randomly fold into the prion conformation. (4) Most importantly, it is not the overexpression of *SUP35* or *URE2* RNA itself that induces prion formation (Derkatch *et al.*, 1996; Masison *et al.*, 1997). The expression of the wild-type protein, or the N-terminal prion-forming domain, is a prerequisite for the propagation of the heritable element (Aigle & Lacroute, 1975; Ter-Avanesyan *et al.*, 1994; Masison & Wickner, 1995; Derkatch *et al.*, 1996; Masison *et al.*, 1997).

Table 1.4 Properties of the yeast prion determinants *[PSI⁺]* and *[URE3]*.

Property	*[PSI⁺]*	*[URE3]*
Non-Mendelian inheritance	✓	✓
Cytoplasmic (transferred by cytoduction)	✓	✓
Reversible curing by GuHCl	✓	✓
Requires expression of a wild-type protein	✓	✓
Overexpression of protein induces *[PRION⁺]*	✓	✓
Same phenotype as chromosomal mutants	✓	✓
Dominant phenotype	✓	✓

1.4 Yeast prion proteins

1.4.1 Yeast prion protein structure

Both Sup35p and Ure2p appear to be fusion proteins, comprising an N-terminal prion-forming domain (PFD) and a 'functional' C-terminal domain (C). The C-domain of Sup35p is homologous to another eukaryotic translation factor, namely elongation factor EF-1α (Kushnirov *et al.*, 1988; Wilson & Culbertson, 1988; Ter-Avanesyan *et al.*, 1994). The C-terminal domain of Ure2p has substantial homology to glutathione-S-transferase (Coschigano & Magasanik, 1991; Masison & Wickner, 1995; Masison *et al.*, 1997). Whilst the PFD's of Sup35p and Ure2p share no obvious sequence homology, both are rich in basic residues and are inessential for the activity of the C-domain (Figure 1.3). Most strikingly, the PFD of Sup35p contains several imperfect repeats of the nonapeptide [PQGGYQQYN] (Kushnirov *et al.*, 1988). The N-terminus of the mammalian prion protein (PrP) also has a series of peptide repeats (in humans - $[PHGGGWGQ]_5$), but it has been proven that this region of PrP is non-essential for the conversion of native (i.e. cellular) protein to a pathogenic isoform (Hope *et al.*, 1988a; Stahl *et al.*, 1993; Fishcer, 1996). Thus, unlike the yeast prion proteins, the N-terminus of PrP cannot be defined as a PFD.

Having established that the expression of the PFD, or the whole prion protein (Sup35p or Ure2p), was necessary for the propagation of the prion-like determinant, it was not immediately obvious how the PFD could influence the activity of the C-domain (Wickner, 1994; Doel *et al.*, 1994; Ter-Avanesyan *et al.*, 1994). It had been proposed that the PFD of Sup35p is a 'trans' acting factor for *[PSI⁺]* maintenance i.e. the PFD interacts with the *[PSI⁺]* determinant to mediate its propagation, but is not the prion itself. Plausibly, this interaction could titrate the 'trans' acting factor, namely Sup35p, away from its normal role in translation termination, giving rise to an allosuppressor phenotype (Doel *et al.*, 1994). However, when expressed in a *[PRION⁺]* background, the C-domains of both Sup35p and Ure2p ablate the prion-associated phenotypes (Ter-Avanesyan *et al.*, 1993, 1994; Wickner *et al.*, 1995, 1997). Thus, the PFD of either

yeast prion protein can only influence the activity of the corresponding C-domain, if they are covalently attached.

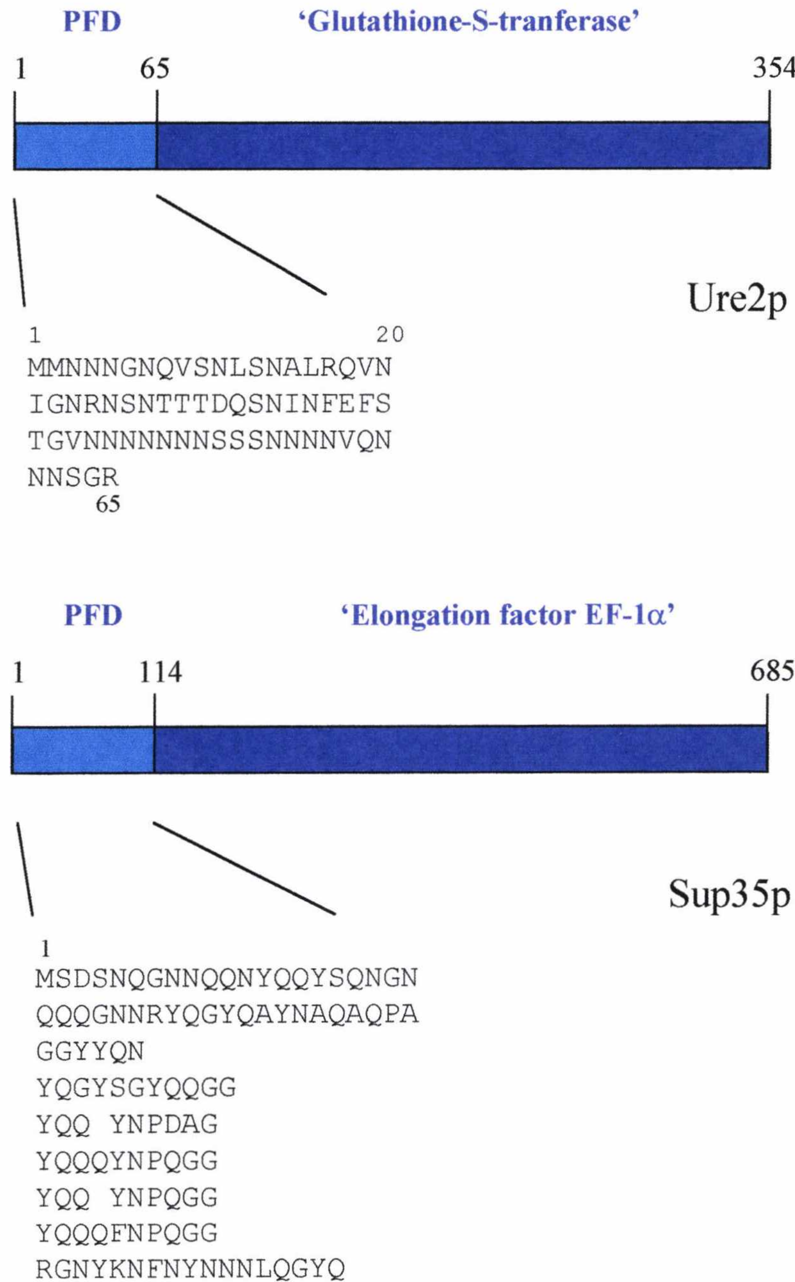


Figure 1.3 Domain structures of Ure2p and Sup35p prion proteins. Expression of the minimal regions, defined as the prion-forming domains (PFD), of Sup35p and Ure2p is essential and sufficient for the propagation of the prion-like determinants *[PSI⁺]* and *[URE3]* respectively (Ter-Avanesyan *et al.*, 1994; Wickner, 1995). The primary sequence of each PFD is shown to illustrate the high content of the basic residues glutamine (Q) and asparagine (N) and the repeat elements of the Sup35p PFD.

A Ure2p PFD-β-galactosidase fusion protein was reported to exhibit no difference in activity, when expressed in a *[URE3]* or *[ure3]* (prion$^-$) background (Masison & Wickner, 1995; Wickner *et al.*, 1997). Intriguingly, the same fusion protein was unable to support *[URE3]*, thereby demonstrating that the PFD was not transferable to an arbitrary protein.

1.4.2 Conformational change of yeast prion proteins *in vivo*

Having demonstrated that the heritable determinants *[PSI$^+$]* and *[URE3]* satisfied the genetic expectations of a prion-like element, Wickner and colleagues pursued biochemical data in support of the yeast prion hypothesis. The mammalian prion had originally been isolated from the brains of scrapie-infected hamsters. Biochemical analysis had suggested that the infectious agent was composed largely, if not exclusively, of a single protein. This protein was designated PrP 27-30 (Bolton *et al.*, 1982; McKinley *et al.*, 1983) and shown to be a truncated product of a normal cellular protein (Oesch *et al.*, 1985). Unlike the cellular protein, PrP^C, the disease-associated protein, termed PrP^{Sc}, possesses a markedly different secondary structure (Pan *et al.* 1993), which is thought to confer resistance to proteolytic degradation.

Masison and Wickner (1995) compared both the quantity and relative protease resistance of Ure2p, isolated from *[URE3]* and *[ure3]* yeast strains. Whilst the intracellular concentration was not different, proteinase K treatment of *[URE3]* extracts gave rise to two stable protein cores, of 33 and 30 kD. Most importantly, these protease-resistant fragments were completely absent from samples of wild-type strains, which raised two possibilities: (1) most probably, the conformation of Ure2p was altered, in a *[URE3]* background, or alternatively, (2) Ure2p was associated with other factors, that conferred protease resistance. A similar enhancement of protease resistance was also reported for the expression of the PFD of Ure2p, in *[URE3]* strains (Masison & Wickner, 1995). So as well as it is unusual genetic properties, *[URE3]* is apparently able to impart a specific structure to the Ure2p.

1.4.3 *[PSI⁺]* requires a modifier of protein structure

Perhaps the most compelling evidence that *[PSI⁺]* is a prion-like determinant, is the requirement for a specific level of the heat-shock protein Hsp104p, for the maintenance of *[PSI⁺]* (Chernoff *et al.*, 1995a). Deletion or overexpression of Hsp104p eliminates *[PSI⁺]*. *In vivo,* Hsp104p conveys stress-tolerance to *S.cerevisiae* by mediating the refolding of stress-denatured proteins (Sanchez & Lindquist, 1990; Sanchez *et al.*, 1992; Parsell *et al.*, 1994a). Hsp104p is essential for *[PSI⁺]* maintenance and as such, *HSP104* is a *PNM* gene (McCready *et al.*, 1977; Chernoff *et al.*, 1995a). It has been proposed that the stress protein catalyses the prion-directed conversion of wild type Sup35p [Figure 1.4] (Chernoff *et al.*, 1995a; Tuite & Lindquist, 1996), since deletion of the *HSP104* gene eliminates *[PSI⁺]*. Conversely, the overexpression of Hsp104p might lead to the 'disaggregation' of the prion template from native Sup35p, thereby precluding the replication of the prion (Chernoff *et al.*, 1995a).

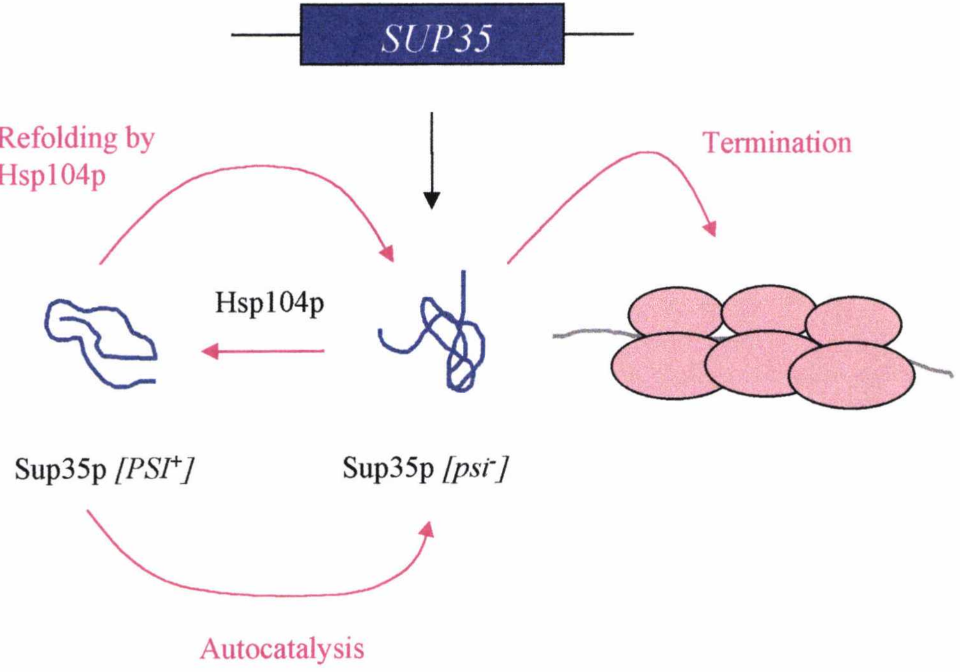


Figure 1.4 'Coprion' and cure (Wickner, 1995). Hsp104p is proposed to convert wild-type Sup35p into the prion-like conformation. Hence, deletion of *HSP104* eliminates the *[PSI⁺]* determinant. Too high a level of Hsp104p (i.e. overexpression) results in renaturation of the Sup35p prion to a native structure and the loss of *[PSI⁺]*.

1.4.4 Properties of Sup35p in a *[PSI⁺]* background

Some thirty years after its discovery, the biochemical characterisation of Sup35p finally revealed the complex relationship between *[PSI⁺]*, the *SUP35* gene and nonsense suppression (Paushkin *et al.*, 1996; reviewed in Tuite & Lindquist, 1996). In support of the yeast prion hypothesis (Wickner, 1994), Sup35p exhibits remarkably different properties when in a *[PSI⁺]* background, compared to those of the wild-type protein. Primarily, $Sup35p^{[PSI+]}$ exists as large intracellular oligomers *in vivo* and is resistant to proteolytic degradation (Paushkin *et al.*, 1996). Unlike PrP^{Sc} or $Ure2p^{[URE3]}$, treatment with proteinase K does not produce a 'stable' fragment of $Sup35p^{[PSI+]}$ (Masison & Wickner, 1995; Paushkin *et al.*, 1996). The intracellular aggregation of Sup35p in *[PSI⁺]* strains has been extensively investigated. For example, the partitioning of Sup35p, between soluble and aggregated forms, has been analysed by centifugation (Paushkin *et al.*, 1996; Patino *et al.*, 1996), gel filtration chromatography (Paushkin *et al.*, 1996) and by microscopic localisation, using green fluorescence tagged-protein (Patino *et al.*, 1996). Each of these techniques has demonstrated that Sup35p exhibits a profound difference, with respect to cellular localisation and oligomeric status, between *[PSI⁺]* and *[psi⁻]* strains.

Sup35p is the yeast polypeptide chain release factor, eRF3, which interacts with at least one other protein, namely Sup45p (eRF1), to mediate translation termination (Stansfield *et al.*, 1995a; Zhouravleva *et al.*, 1995). The demonstration that the *[PSI⁺]* determinant is Sup35p (Chernoff *et al.*, 1993; Doel *et al.*, 1994; Ter-Avanesyan *et al.*, 1994) and that $Sup35p^{[PSI+]}$ has an inherent propensity to oligomerise (Patino *et al.*, 1996; Paushkin *et al.*, 1996) affords a rational explanation of the allosuppressor phenotype of *[PSI⁺]* strains. In a *[PSI⁺]* background, the cell is depleted of soluble Sup35p, by virtue of its propensity to aggregate. This results in a decreased translation termination efficiency, since the intracellular concentration of functional release factor complex (eRF1 + eRF3) is reduced (Paushkin *et al.*, 1996, 1997b). The genetic properties of the *[PSI⁺]* determinant (i.e. dominant and extrachromosomal) reflect the ability of a cytoplasmic *[PSI⁺]* prion, to interact with wild-type Sup35p and promote its conversion to the same non-functional conformation (Figure 1.5).

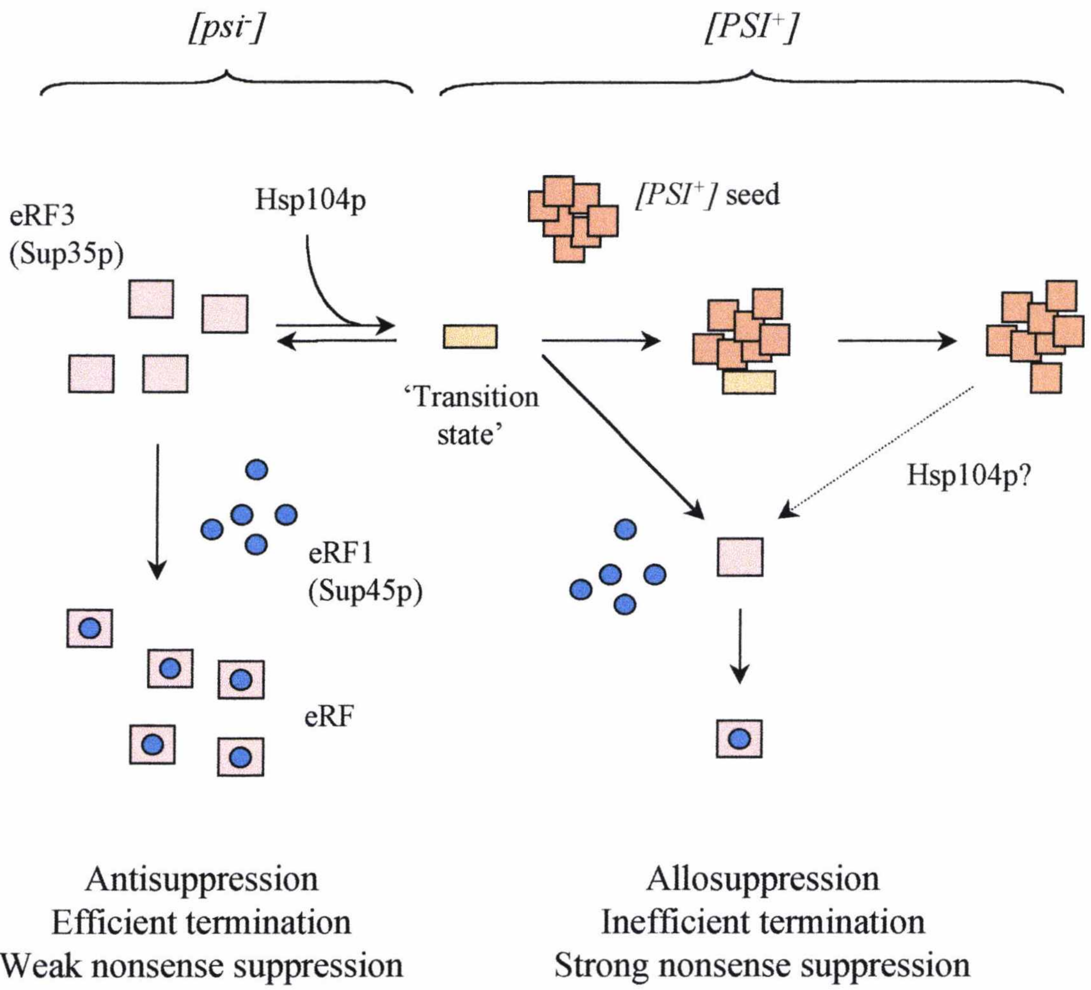


Figure 1.5 A model for the relationship between the prion-like protein Sup35p, Hsp104p, the heritable determinant *[PSI+]* and its allosuppressor phenotype (as described by Tuite & Lindquist, 1996). Hsp104p facilitates the newly synthesised Sup35p (eRF3) molecules to attain a protein-folding transition state, which is a prerequisite for the formation of the *[PSI+]* prion form of Sup35p. If the prion forms of Sup35p are present, they capture this conformer, sequestering Sup35p from the ribosomes and/or its binding partner eRF1 (Sup45p) and propagating the aggregated prion form. If the *[PSI+]* prion form of Sup35p is not present, the transition state is unstable and reverts to the native *[psi-]* form of the protein, which is then able to participate in translation termination. In *[PSI+]* cells, a low level of functional eRF is present using Sup35p molecules that either escape sequestration or are generated by the disaggregation of the Sup35p aggregates.

1.5 Prion biology and disease

The yeast prion hypothesis (Wickner, 1994) offers a satisfactory explanation of the unusual genetic properties of the non-Mendelian heritable elements *[PSI$^+$]* and *[URE3]* (see Chapter 1.3.2). The biophysical analysis of the two putative yeast prion proteins, Sup35p and Ure2p respectively, supports the notion that heritable phenotypes can be 'encrypted' within protein structures in yeast (reviewed in Wickner *et al.*, 1996; Tuite & Lindquist, 1996; Lindquist, 1997). The study of yeast prions has obviously drawn upon the experience and knowledge already gained with mammalian prion research. The similarities between the two areas of prion research are illustrated below.

1.5.1 Prion diseases

The first spongiform encephalopathy was characterised in sheep, as the disease scrapie. The transmissibility of the disease was dramatically illustrated, when a flock of sheep was inoculated with a formalin-treated extract of contaminated lymphoid tissue. Nearly 10% of those 'inoculated' animals went on to develop scrapie (Gordon, 1946). Gajdusek subsequently identified 'kuru' as a human form of the disease, which was transmitted by ritual cannibalism, in highlanders of New Guinea (Gajdusek & Zigas, 1959; Gajdusek, 1977). The brains of kuru-victims were found to have protein-rich 'plaque' lesions, which were shown to contain the transmissible agent. This was demonstrated by the cerebral inoculation of chimpanzees, with extracts of kuru brain (Gajdusek *et al.*, 1966). To date, at least four other related, human neurodegenerative, diseases have been described; Gerstmann-Straüssler-Scheinker (GSS) disease, fatal familial insomnia (FFI), fatal sporadic insomnia (FSI) and Creutzfeldt-Jacob disease (CJD) (reviewed in Prusiner *et al.*, 1998). As demonstrated by CJD, the disease can arise spontaneously (i.e. sporadic, sCJD), can be inherited (i.e. familial, fCJD), or transmitted iatrogenically, through transplantation of infected tissues (iCJD) or most notoriously, by the apparent ingestion of meat products contaminated with the BSE agent (new variant, vCJD).

The remarkable properties of the scrapie agent, such as resistance to ionising radiation (Alper *et al.*, 1967), prompted numerous suggestions as to its nature (reviewed in Pruisner, 1982), but it was Griffith (1967) who first hypothesised that the scrapie agent could be a self-replicating protein. Diseased animals typically, but not universally, accumulate extracellular deposits of protein, termed plaques, within their central nervous systems (Merz *et al.*, 1981; Hope *et al.*, 1988b). The principal component of plaques is a single protein (Diringer *et al.*, 1983; Prusiner *et al.*, 1980, 1984), that is present as both amorphous aggregates and as ordered structures, known as scrapie-associated fibrils (SAF). When recovered from tissues, these rod-like fibres typically measure 10-25nm x 100-200nm and are associated with scrapie infectivity (Merz *et al.*, 1981; Diringer *et al.*, 1983; Prusiner *et al.*, 1983; Hope *et al.*, 1988b; McKinley *et al.*, 1991). The term prion was used to describe the protein component of SAF, which is required for infectivity (Prusiner, 1982). The protein-only, or prion, hypothesis holds that the prion is a self-replicating particle, devoid of a nucleic acid and that the essential infectious component is a protein. Whilst prions are defined as proteinaceous and as such, have been described as infectious proteins (Pan *et al.*, 1993; Telling *et al.*, 1995), it remains unknown whether there exists an associated ligand, that is required for prion propagation (Prusiner *et al.*, 1998).

All forms of prion disease are associated with the altered metabolism of a host-encoded cellular protein, namely PrP^{C} (Prusiner *et al.*, 1984; Chesebro *et al.*, 1985; Oesch *et al.*, 1985; Prusiner, 1991). Unlike PrP^{C}, the disease-associated protein PrP^{Sc} is insoluble in nondenaturing detergents and exhibits significant resistance to proteolytic degradation (Oesch *et al.*, 1985; Meyer *et al.*, 1986). Given that PrP^{C} and PrP^{Sc} share the same primary structure (Basler *et al.*, 1986), it was proposed that the conversion of the normal protein involved either a conformational change or a post-translational modification (Hope *et al.*, 1986). Whilst it was been reported that there is no covalent difference between PrP^{C} and PrP^{Sc} (Stahl *et al.*, 1993), Pan *et al.* (1993) demonstrated that PrP^{C} undergoes a significant rearrangement of secondary structure, upon conversion into the prion form. On the basis of Fourier transform infrared spectroscopy (FTIR) and circular dichroism (CD) measurements, PrP^{C} is ~40% α-helical, whereas the protease resistant core of PrP^{Sc} is ~50% β-sheet and only ~20% α-helix (Caughey *et al.*, 1991; Pan *et al.*, 1993; Safar *et al.*, 1993). The structures of several recombinant PrP molecules have

recently been solved, by nuclear magnetic resonance spectroscopy (Riek *et al.*, 1996; Donne *et al.*, 1997; James *et al.*, 1997) and confirm the structural 'predictions' for PrP^C by FTIR and CD. The C-terminal portion of PrP comprises 3 α-helices and 2 short stretches of anti-parallel β-strands, which have been suggested to form a nucleus, for the conversion of the native structure to the pathological conformation (Riek *et al.*, 1996).

1.5.2 Genetics of prion diseases

The major component of the scrapie prion, PrP^{Sc}, is the product of a single host-encoded gene, *Prnp* (Oesch *et al.*, 1985). Basler *et al.* (1986) demonstrated that there was no difference in primary structure of PrP isolated from a normal animal, or that predicted from the cDNA from a scrapie-infected animal. Furthermore, levels of PrP mRNA are found to be the same in both infected and non-infected animals (Chesebro *et al.*, 1985; Oesch *et al.*, 1985). In support of the notion that endogenous PrP was disease-associated, a genetic predisposition was identified in families with GSS, FFI and fCJD. The *Prnp* genes from these family members were shown to encode specific amino acid substitutions or polymorphisms, which lead to the onset of disease (Prusiner, 1996). To date, more than 20 different mutations in the *Prnp* gene are known to cause inherited human spongiform encephalopathies, with significant genetic linkage having been established for 5 of these (Hsiao *et al.*, 1989; Prusiner, 1996). Similarly, in mice, *Prnp* has been genetically linked to the locus controlling scrapie incubation times [i.e. the time before the onset of disease, following inoculation] (Dickinson *et al.*, 1968; Carlson *et al.*, 1986, 1994; Scott *et al.*, 1997; Moore *et al.*, 1998).

Transgenic mice have permitted several experiments that are suggested to provide irrefutable evidence that the scrapie agent is a conformational variant of the cellular protein PrP. First, deletion of the *Prnp* gene confers resistance to scrapie and ablates the generation of nascent infectious particles (Büeler *et al.*, 1993; Prusiner *et al.*, 1993). Second, transgenic mice expressing a mutant PrP, that bears a GSS-associated substitution, P102L, develop a spontaneous neurodegenerative disease (Hsiao *et al.*, 1990, 1994; Telling *et al.*, 1996b). Studies with transgenic mice have also demonstrated that the infecting prion acts as a 'template' for the conversion of the cellular protein.

There appears to be a direct interaction between the prion template and its substrate, which results in the faithful reproduction of the 'infective' conformer (Prusiner *et al.*, 1990). Moreover, this interaction and hence the 'replication' of the infectious prion, is inhibited by differences of sequence, that exist between template and substrate PrP molecules. The species barrier is a reflection of the specificity of prion 'templating'.

Generally, one species is resistant to infection by prions developed in another species. This phenomenon is referred to as the 'species barrier'. 'The species of a particular prion is encoded by the sequence of the chromosomal *Prnp* gene of the mammal in which it last replicated' (Prusiner *et al.*, 1998). Until recently, this barrier was thought to have prevented the infection of higher primates with ungulate (e.g. bovine) prions (Bruce *et al.*, 1996; Collinge *et al.*, 1996). Scott *et al.* (1989) demonstrated that the species barrier could be 'overcome' by the expression of a heterologous *Prnp* gene. Mice are normally resistant to the hamster prion, but transgenic mice that expressed the hamster prion, became susceptible to infection with hamster prions. Within the framework of the protein-only hypothesis, there should be a direct interaction between the infectious PrP^{Sc} and the endogenous PrP^{C}. The degree of 'corruption' of the endogenous PrP^{C} will depend upon the significance of any difference in primary structure, between the PrP sequences. In contrast to pathogens that bear nucleic acid and exhibit gene-encoded properties, the species-specific properties of prions appear to be encrypted within the three dimensional structure of PrP^{Sc} (Prusiner *et al.*, 1998).

1.5.3 Prion strains and variation in yeast phenotypes

Like the mammalian prion PrP^{Sc}, the yeast determinants *[PSI⁺]* and *[URE3]* are believed to reflect the properties of an alternative conformer of a host-encoded protein. In all three cases, the expression of the chromosome-encoded protein is a prerequisite for the propagation of the prion (Aigle & Lacroute, 1975; Büeler *et al.*, 1993; Prusiner *et al.*, 1993; Ter-Avanesyan *et al.*, 1994; Derkatch *et al.*, 1996; Masison *et al.*, 1997). Overexpression of the normal cellular protein increases the frequency of spontaneous appearance of the prion (Scott *et al.* 1989, Prusiner *et al.* 1990, Chernoff *et al.* 1993, Wickner 1994). Clearly, these genetic data are entirely consistent with the prion

hypothesis. Furthermore, the increased resistance to proteolytic degradation, exhibited by all of the putative prion proteins in a *[PRION$^+$]* background, provides biochemical evidence in support of a protein-conformation mediated phenomenon (Oesch *et al.*, 1985; Wickner, 1994; Paushkin *et al.*, 1996).

Another feature common to scrapie and *[PSI$^+$]*, is that of strain variation. Different preparations of scrapie-infectious material, isolated from different sources, can give rise to different scrapie 'strains'. Individual strains are able to elicit a distinct and reproducible incubation period, pattern of neurodegeneration and profile of protease resistant cores (Dickinson *et al.*, 1968; Fraser & Dickinson, 1968; Bruce *et al.*, 1989; DeArmond *et al.*, 1997). What is more, all of these strain variants can be propagated in the same strain of mouse, suggesting that the same polypeptide can convey different strain phenotypes. Using transgenic mice that expressed a chimeric human-mouse PrP protein, it was demonstrated that the same PrP protein could indeed, support two different prion strains (Telling *et al.*, 1996). Two groups of the transgenic mice were inoculated with two distinct scrapie strains and ultimately yielded two different patterns of protease-resistant PrP^{Sc} fragments, thereby demonstrating that the 'phenotype' of two distinct strains can be determined, by two different protein conformations, of the same polypeptide.

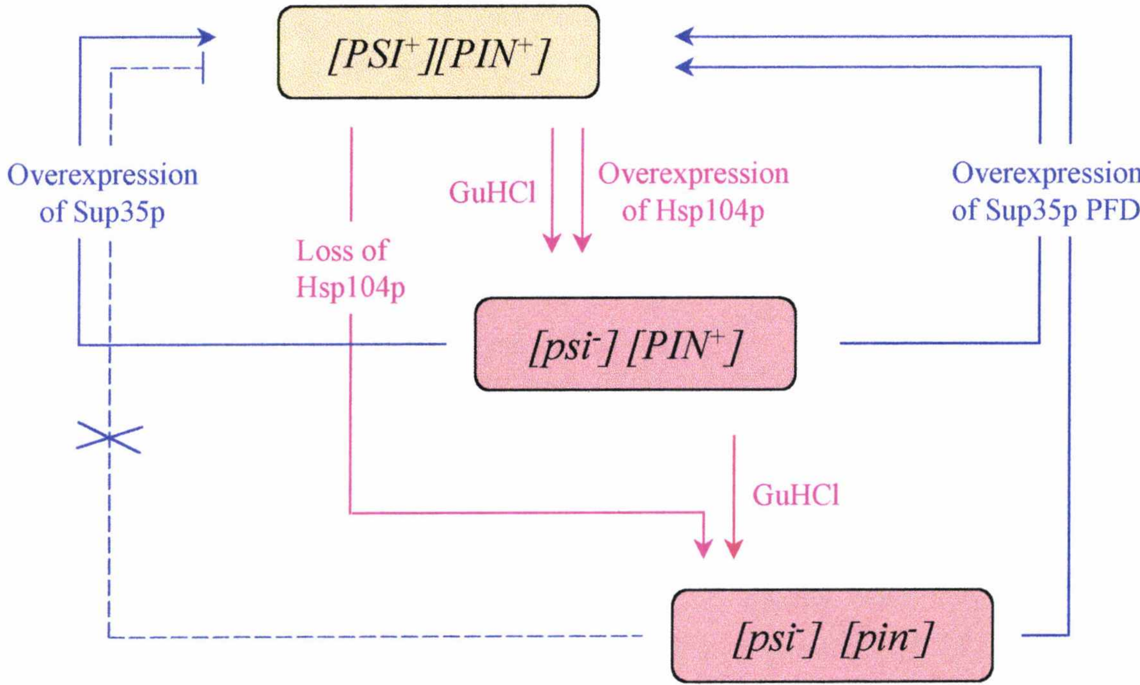
The propagation of strain characteristics, in the form of stable variations of PrP structure, has also been demonstrated *in vitro*, using a cell-free conversion assay (Bessen *et al.*, 1995). The diversity of prion strains requires an equal diversity of PrP structure. Such complexity could be seen to arise if, for example, the prion is a multimeric structure (Lansbury & Caughey, 1995; Prusiner, 1996). Strains might represent different packing configurations of monomers, which could result from 'seeds' of different size. The incubation period of a particular strain might reflect the rate of polymer growth and the pathological properties, associated with that specific pattern of aggregation. Alternatively, other factors such as glycosylation could introduce a vast degree of strain diversity (Aguzzi & Weissmann, 1996; Collinge *et al.*, 1996), although they may not be required for conversion to a pathological conformation (Taraboulos *et al.*, 1990).

In yeast, there is also considerable evidence of prion strains and conformation-encrypted, phenotypic variation. The overexpression of Sup35p can elicit the *de novo* generation of phenotypically distinguishable *[PSI⁺]* determinants, within the same strain (Derkatch *et al.*, 1996). These *[PSI⁺]* elements can elicit either a 'weak' or a 'strong' allosuppressor phenotype (i.e. inefficient translation termination) and furthermore, they also differ with respect to their mitotic stabilities. One such variant of *[PSI⁺]*, termed *[ETA⁺]*, was originally identified by its lethality, when in the presence of certain *SUP35* or *SUP45* mutant alleles (Liebman & All-Robyn, 1984). Like *[PSI⁺]* and *[URE3]*, it can be eliminated by growth in the presence of GuHCl, but *[ETA⁺]* segregates irregularly during meiosis (Liebman & All-Robyn, 1984). *[ETA⁺]* can be induced by the overexpression of *SUP35* and requires Hsp104p for its propagation. Furthermore, Sup35p is present as aggregates in *[ETA⁺]* strains of yeast, but not in isogenic *[eta⁻]* strains, demonstrating that *[ETA⁺]* is also a manifestation of the prion-like properties of Sup35p (Zhou *et al.*, 1998).

It has recently become apparent that the conformation of Sup35p may actually 'encode' a second self-propagating phenotype, in addition to *[PSI⁺]*. The elimination of *[PSI⁺]*, by growth on media containing GuHCl, had been reported as both reversible and irreversible (Tuite *et al.*, 1981; Cox, 1988; Chernoff *et al.*, 1993, 1995). Curing by GuHCl can however produce two 'types' of *[psi⁻]* strain (Derkatch *et al.*, 1997). These two forms are differentiated by their ability to revert to a *[PSI⁺]* status, upon overexpression of full-length Sup35p (Figure 1.6). Those strains that can revert are designated *[PIN⁺]* (for Prion-INducible), whereas those that do not exhibit *de novo* *[PSI⁺]*-induction are denoted *[pin⁻]* (Derkatch *et al.*, 1997). *[PSI⁺]* can be induced *de novo*, in both *[PIN⁺]* and *[pin⁻]* variants of *[psi⁻]* strains, by overexpression of the N-terminus of Sup35p (residues 1-154).

Whilst *[PIN⁺]* is not essential for *[PSI⁺]* maintenance (Bradley *et al.*, 1998), it appears that the *de novo* formation of a self-propagating prion conformer (i.e. prion seed) of full-length Sup35p, does require the presence of *[PIN⁺]* (Derkatch *et al.*, 1997). Like *[PSI⁺]*, *[PIN⁺]* is susceptible to curing by GuHCl and by deletion of Hsp104p, but *[PIN⁺]* is not lost upon overexpression of Hsp104p. Genetic studies demonstrated that *[PIN⁺]* is independent of the prion-forming domain of Sup35p and that this

'inducibility' factor is also a cytoplasmic, prion-like determinant. These data have lead to the hypothesis that *[PIN⁺]*, actually reflects a variation of conformation, within the C-terminal domain of Sup35p (Derkatch *et al.*, 1997). These observations imply that in yeast, Sup35p is not only capable of supporting different 'strains' of the same prion (e.g. 'weak' and 'strong' *[PSI⁺]*), but that the same polypeptide actually bears two distinct prion-like domains (i.e. *[PSI⁺]* - N-terminal domain, *[PIN⁺]* – C-terminal domain).



<u>Figure 1.6</u> Sup35p may possess two separate prion-like domains, whose behaviour generates two distinct phenotypes, *[PSI⁺]* and *[PIN⁺]* (Derkatch *et al.*, 1997). GuHCl-induced elimination of *[PSI⁺]* produces two *[psi⁻]* variants, designated *[PIN⁺]* and *[pin⁻]*, that differ with respect to *[PSI⁺]*-induction, upon overexpression of the full-length Sup35p. The *[PSI⁺]* determinant is the N-terminal prion-forming domain (PFD) of Sup35p, whereas *[PIN⁺]* is proposed to be a second prion-like determinant, encoded by the C-terminus of Sup35p. Like *[PSI⁺]*, the *[PIN⁺]* element requires Hsp104p activity for maintenance and is 'cured' by GuHCl or deletion of the *HSP104* gene.

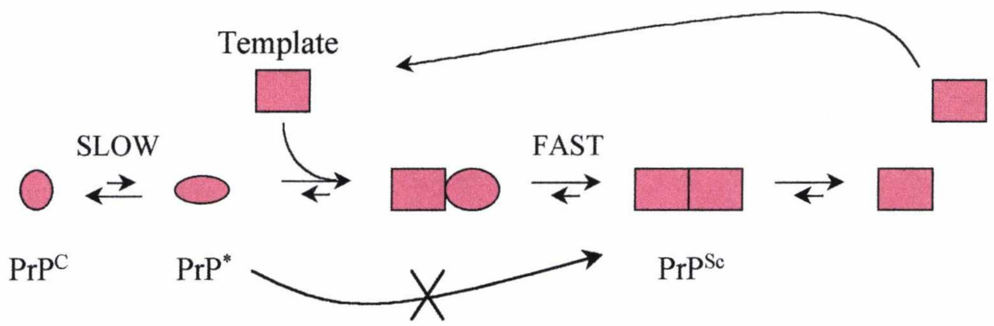
1.6 Prion protein folding

1.6.1 Mechanism of prion replication

Biochemical analysis of scrapie-associated plaques has implicated a conformational variant of PrP, as the key factor in the pathogenesis of spongiform encephalopathies (Prusiner *et al.*, 1982; Meyer *et al.*, 1986; McKinley *et al.*, 1993; Pan *et al.*, 1993). Studies with transgenic mice have suggested a direct interaction between the prion template and its substrate, which results in the faithful reproduction of the pathogenic conformer (Prusiner *et al.*, 1990). Two models have been proposed for the mechanism of prion replication. Both models permit a strain-specific conformation (and of course its replication), but differ with respect to the nature of the prion template and the 'slow' step in the process of prion replication. In the 'template assistance' model, the rate-limiting step is the actual refolding of PrP^{C} into the PrP^{Sc} conformation (Prusiner, 1991; Cohen *et al.*, 1994; reviewed in Horwich & Weissman, 1997). PrP^{Sc} is considered to be a thermodynamically favoured, but kinetically inaccessible form of PrP^{C}. *In vivo,* PrP^{Sc} is proposed to bind to PrP^{C} or PrP*, a partially unfolded form of PrP^{C}, thereby lowering the activation energy barrier between the cellular and pathogenic conformers and accelerating the production of PrP^{Sc} (Figure 1.7). Pathological mutations in PrP are thought to destabilise the cellular protein, thereby favouring a PrP^{C}/PrP* to PrP^{Sc} conformational change. Interconversion is still a rare event, thereby offering an explanation for the late-onset of the familial encephalopathies (Weissmann, 1994).

The 'nucleation-dependent polymerisation' (NDP) model stipulates that the rate-limiting step in prion propagation, is the formation of a stable multimeric complex of PrP^{Sc} (Jarrett & Lansbury, 1992, 1993). The interconversion of PrP^{C} and PrP^{Sc} conformers is proposed to be facile, but monomeric PrP^{Sc} is less thermodynamically favoured, than monomeric PrP^{C}. However, an ordered aggregate of PrP^{Sc} is thermodynamically satisfied and able to catalyse the 'fast' conversion of PrP^{C} to PrP^{Sc} (Come *et al.*, 1993; Jarrett & Lansbury, 1993). The slow step of the NDP model is the actual formation of a stable PrP^{Sc} nucleus, or 'seed' (Figure 1.7). Once the nucleus has

formed, polymerisation is thermodynamically favoured (i.e. fast) as monomers form multiple, stabilising, interactions with the growing oligomers.



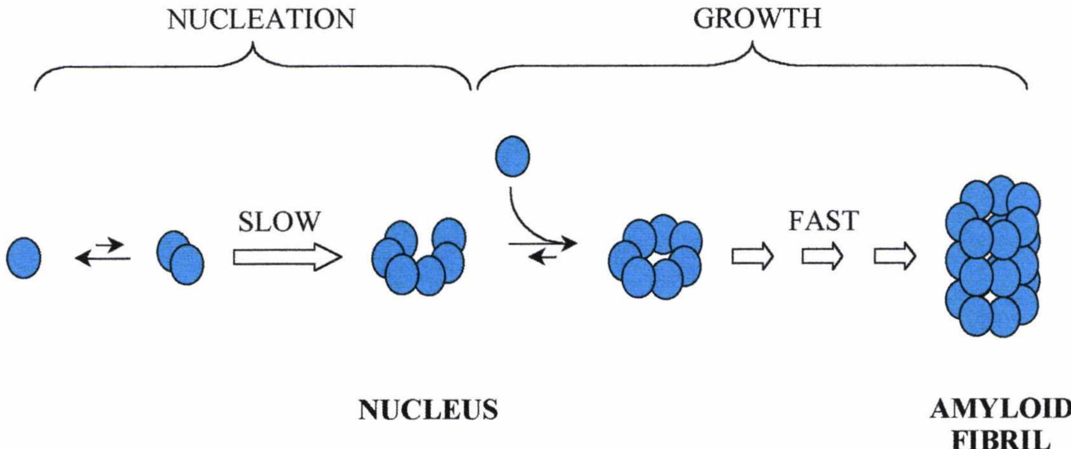


Figure 1.7 Models for the propagation of the pathogenic prion conformation. (1) In the template assistance model (Prusiner, 1982, 1991), the interconversion of monomer and 'template conformer' is thermodynamically unfavoured (i.e. slow). The spontaneous formation of template is first order with respect to monomer concentration, but is extremely rare. However, if formed *de novo* or introduced from an external source, a template is proposed to accelerate the rate of monomer conversion and template-replication proceeds rapidly. **(2)** The interconversion of monomer and a polymer-competent conformer is facile in the nuclear dependent polymerisation (NDP) model (Jarrett & Lansbury, 1992, 1993). The rate-limiting step is nucleus formation and the rate of NDP depends upon the $[\text{monomer}]^n$, where n = minimum number of monomers in a stable nucleus. However, this slow step can be overcome by 'seeding' with pre-formed nuclei, an event that permits the rapid polymerisation of monomer.

In a NDP reaction, there will be a critical monomer concentration, below which seed formation is precluded. The kinetics of a NDP event is predicted to exhibit a lag phase, which reflects the kinetic barrier to seed formation (Figure 1.8). Once nuclear formation has occurred, polymer growth (i.e. loss of monomer) is 'fast' and thermodynamically favoured. Whilst the duration of a nucleation lag is extremely sensitive to monomer concentration, the nucleation lag can be eliminated by 'seeding' a system with pre-formed fibrils (i.e. an external source of nuclei). Seeding initiates the rapid thermodynamic-favoured growth of polymer.

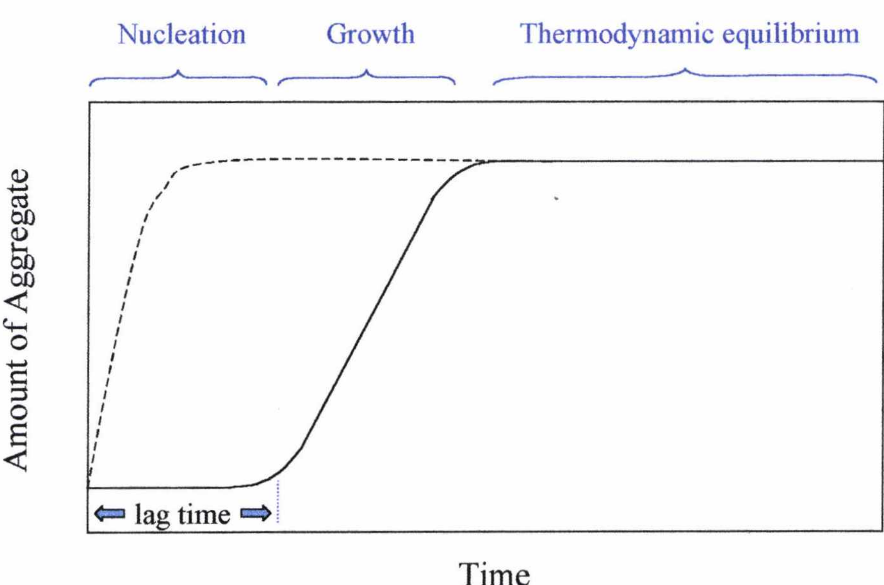


Figure 1.8 Profile of nucleation-dependent aggregate formation, above the critical monomer concentration (Jarrett & Lansbury 1993). The solid line indicates aggregate formation. At high concentration, nucleation is so rapid that no lag time is observed (dashed line). Similarly, the kinetic barrier to polymer growth can be overcome by the addition of a preformed nucleus, termed 'seeding', which also instigates rapid growth (dashed line).

Both template-assisted and NDP models offer attractive explanations for the behaviour of prions *in vivo* (Harper & Lansbury, 1997; Horwich & Weissman, 1997; Prusiner *et al.*, 1998). The most contentious issue seems to be whether infectious PrP^{Sc} is polymeric. Crude extracts of scrapie-infected brains have been reported to contain fibrillar structures, termed scrapie-associated fibrils [SAF] (Merz *et al.*, 1981; Diringer *et al.*, 1983; McKinley *et al.*, 1991). The full-length PrP molecule is the major component of SAF *in vivo* (Hope *et al.*, 1986) and yields the protease-resistant core

PrP27-30, *in vitro* upon detergent-extraction and limited proteolysis (Prusiner *et al.*, 1983; McKinley *et al.*, 1991; Wille *et al.*, 1996). Various methods of preparation have yielded small rod-like aggregates, termed prion rods, which bind Congo red dye and exhibit green-gold birefringence under polarised light (Prusiner *et al.*, 1983). These properties are typical of amyloid, which is composed of ordered filamentous protein polymers, arranged in a 'cross β' repeat structure in which the β strands are aligned perpendicular to the axis of the amyloid fibril. (Glenner *et al.*, 1972; Glenner, 1980). The NDP model proposes that prion replication proceeds via the addition of PrP^{C} monomer to seeds of PrP^{Sc} amyloid (Jarrett & Lansbury, 1993; Bessen *et al.*, 1995; Caughey *et al.*, 1995).

There are several lines of evidence that contest the role of amyloid and indeed the NDP model, in scrapie-type diseases (Prusiner *et al.*, 1998). Amyloid formation and PrP^{Sc} aggregation *per se* are not always associated with spongiform encephalopathies (Hsiao & Prusiner, 1994). Structures associated with infectivity and amyloid-formation may exhibit different dye-binding properties and macrostructure, as determined by electron microscopy (Wille *et al.*, 1996). It has been reported that a significant proportion of total PrP can be separated from infectivity (Manuelidis *et al.*, 1987). Whilst this was construed as evidence against both the NDP model and the protein-only hypothesis itself, it could also be interpreted as evidence that polymerisation (i.e. amyloidosis) and infectivity do actually constitute different phenomena. The scrapie agent is resistant to deactivation by ultraviolet and ionising radiation, a treatment that usually destroys nucleic acid (Alper *et al.*, 1966, 1967). These and other studies have suggested that the infectious agent is small and probably a dimer (Alper *et al.*, 1966; Bellinger-Kawahara *et al.*, 1988). These data are clearly more consistent with a template-assisted mechanism of prion replication, rather than that of nuclear-dependent polymerisation.

It has been argued that an increased infectivity, generated by the disruption of amyloid fibrils, is consistent solely with the template assistance model (Gabizon *et al.*, 1987). However, the fragmentation of prion 'amyloid' is recognised as a prerequisite for prion propagation, (Orgel, 1996) and has been demonstrated experimentally, *in vitro* (Caughey *et al.*, 1995, 1997; DePace *et al.*, 1998). Indeed, those fragments that could support prion conversion *in vitro,* were themselves too large to be dimers (Caughey *et*

al., 1995). It is much easier to visualise certain aspects of 'prionology' within the framework of the NDP model. For example, the large number of prion strains can be readily envisaged as a reflection of a huge variety of nucleated polymers (Westaway *et al.*, 1995; Lansbury & Caughey, 1995). Nuclei could vary with respect to n (the minimal number of monomers required for thermodynamic stability of a nucleus), packing order and configurations of monomers. Inherited and sporadic encephalopathies would reflect mutations that promote monomer-monomer interactions and thereby favour seed formation (Lansbury & Caughey, 1995). Such a precedent is found with haemoglobin S and sickle cell disease, in which the nucleated-polymerisation of the mutant protein occurs within red blood cells (Hofrichter *et al.*, 1974). Whilst it appears that certain researchers have a strong preference for either the template assistance model (Prusiner *et al.*, 1998), or the NDP scheme (Lansbury & Caughey, 1996), there are several key issues that are equally valid for both hypotheses. In agreement with both of their respective kinetic schemes (Figure 1.7), the models propose that the introduction of a prion seed (either by inoculation or *de novo* formation) permits the circumvention of the 'slow' step in the conversion of PrP^{C} to PrP^{Sc}. Thereafter, the sequestration of PrP^{C} proceeds rapidly, as reflected in the 'all or nothing' of the spongiform encephalopathies.

In vivo, formation of PrP^{Sc} is believed to be a post-translational process (Borchelt *et al.*, 1990) that occurs, as PrP^{C} is recycled from the cell surface, via the endocytic pathway (Caughey & Raymond, 1991; Gorodinsky & Harris, 1995; Taraboulos *et al.*, 1995). Conceivably, the concentration of PrP^{C} within surface-associated structures (i.e. calveolae) or within an intracellular structure (i.e. endosomes) could accelerate the rate of prion formation. Recombinant PrP exists as a partially unfolded structure at pH 4 and is rich in β-sheet (Swietnicki *et al.*, 1997; Hornemann and Glockshuber, 1998). Plausibly, this structure might occur within the acidic environment of the endosome and as such, could represent an intermediate in the conversion of PrP^{C} to PrP^{Sc}. *In vitro* conversion of PrP^{C} is enhanced by the pre-treatment of PrP^{Sc} with 2-3M GuHCl (Caughey *et al.*, 1995). Thus, it appears that prion-replication may be accelerated *in vivo,* by a destabilisation of either PrP^{C} or PrP^{Sc}.

The role of ancillary factors (other than nucleic acid!) in the replication of the prion, is an idea fully supported by both models (Prusiner *et al.*, 1998; Harper & Lansbury, 1997). There is increasing evidence that cellular factors, such as cholesterol and sulfated proteoglycans, exert a profound effect upon prion replication *in vivo* (Caughey & Raymond, 1993; Taraboulos *et al.*, 1995). As an attractive alternative, PrP^{C} might interact with an endogenous factor, such as a protein chaperone, that directly mediates changes in its structure (Telling *et al.*, 1995; Kenward *et al.*, 1996; Welch & Gambetti, 1998). It has even been suggested that PrP^{Sc} may itself function as a chaperone (Liautard, 1991).

The strongest evidence for the interaction of a prion protein with a chaperone, is found in yeast, with the requirement of the heat shock protein Hsp104p, for the maintenance of the *[PSI^{+}]* determinant (Chernoff *et al.*, 1995a; see Chapter 1.4.3). Molecular chaperones perform a variety of tasks in the cell, but essentially function as modifiers of protein structure (reviewed in Ellis, 1998; Netzer & Hartl, 1998). Chaperones bind to newly synthesised polypeptides and guide their folding and assembly, they promote translocation of proteins into organelles and protect the cell against environmental stress. Scrapie infection has been reported to modulate the intracellular levels of heat shock proteins (Kenward *et al.*, 1994; Tatzelt *et al.*, 1995), although there is no direct evidence of a role for chaperones in the propagation of PrP^{Sc}. However, studies with transgenic mice have revealed the presence of a putative species-specific macromolecule, that participates in the replication of mammalian prions (Telling *et al.*, 1995). Transgenic mice expressing human (Hu) PrP are largely resistant to infection with Hu prions (Telling *et al.*, 1994). However, homozygous knockout mice (*$Prnp^{0/0}$*) expressing only HuPrP are extremely senstive to Hu prions, thereby demonstrating that the mouse (Mo) PrP in some way inhibits the replication of Hu prions, when co-expressed with HuPrP. It is proposed that MoPrP blocks the conversion of HuPrP to $HuPrP^{Sc}$, by preferentially binding to an endogenous factor, designated protein X, that is required for prion propagation [Figure 1.8] (Telling *et al.*, 1995). Whilst these data implicate an endogenous factor in prion formation, the putative protein X remains unidentified.

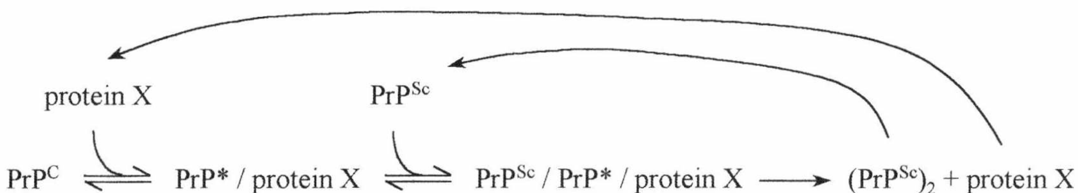


Figure 1.8 Template-assisted conversion of PrP^C by protein X and PrP^{Sc} (Prusiner *et al.*, 1998). The template assistance model has been modified to incorporate the action of the putative protein X (Telling *et al.*, 1995). Initially PrP^C binds to protein X, forming a PrP* / protein X complex. PrP^{Sc} then binds to the PrP* / protein X complex. When PrP* 'refolds' to yield nascent PrP^{Sc}, protein X dissociates from the PrP^{Sc} dimer. A fraction of PrP^{Sc} dimers dissociate to yield 'template' monomers, thereby ensuring successive rounds of PrP^C conversion.

1.6.2 Studying prion formation *in vitro*

The use of transgenic mice has permitted an assessment of the relationship between *Prnp* gene expression and the rate of disease development (Prusiner *et al.*, 1990, 1993; Büeler *et al.*, 1993; Carlson *et al.*, 1994). However, this biological assay has failed to provide the same understanding of prion formation, as that gained from experiments that have followed the *in vitro* conversion and polymerisation of purified prion proteins (reviewed in Harper & Lansbury, 1997). Kocisko *et al.* (1994) first demonstrated the cell-free conversion of purified hamster PrP^C to a protease-resistant, PrP^{Sc}-like conformation. The *in vitro* system comprised isolated PrP^{Sc}, radiolabelled affinity purified PrP^C and anti-PrP^C antibody. Following the disruption of the PrP^{Sc} isolate, by treatment with 3M GuHCl, PrP^{Sc} converted PrP^C to a proteinase K–resistant conformer. It was demonstrated that PrP^{Sc} structure was essential to the conversion process, since the isolation of PrP^{Sc} under more severe conditions (i.e. 3.5M GuHCl at 37°C) eliminated converting activity (Kocisko *et al.*, 1994). Moreover, whilst GuHCl is known to solubilise a large proportion of aggregated PrP^{Sc}, converting activity is only observed in sedimentable fractions, regardless of GuHCl concentration (Caughey *et al.*, 1995, 1997). Despite the apparent demonstration of 'protein-only' replication *in vitro*, the cell-free system does have two severe limitations: a large excess of PrP^{Sc} is required

to promote the conversion of PrP^C and to date, it has not been possible to demonstrate the generation of nascent infectivity.

The cell-free conversion assay has also been employed to address the molecular basis of the scrapie species barrier (Bessen *et al.*, 1995; Kocisko *et al.*, 1995). *In vitro,* mouse PrP^C was converted by mouse PrP^{Sc} and similarly, hamster prions catalysed the corruption of the homologous PrP^C. Whilst mouse prions converted hamster PrP^C, the reciprocal reaction did not occur. These data demonstrate that species specificity exists at the molecular level (i.e. PrP primary sequence) and were in complete agreement with the relative transmissibilities of these scrapie strains, between mice and hamsters *in vivo.* Furthermore, the cell-free system has also permitted the demonstration that the pathological difference between scrapie strains, is mirrored by a change in PrP^{Sc} structure (Bessen *et al.,* 1995). PrP^{Sc} from two distinct prion strains catalysed the reproducible *in vitro* conversion of PrP, into two different conformations (as determined by proteinase K resistance).

The kinetics of the cell-free prion formation reactions were consistent with a nucleation-dependent polymerisation process (Jarrett & Lansbury, 1993; Caughey *et al.,* 1995). In each case, the formation of protease-resistant PrP did not occur below a threshold (critical) concentration of PrP^C and the conversion was initiated by seeding, with fragmented PrP^{Sc}. The propensity of PrP fragments to polymerise, into amyloid fibres, has also permitted the *in vitro* assessment of the consequences of a non-pathological polymorphism of *Prnp* (Come *et al.,* 1993; Come & Lansbury, 1994). The polymorphism occurs at position 129 in PrP and comprises a valine for methionine substitution. The homozygous genotype confers a predisposition to both sporadic and infectious CJD (Palmer *et al.,* 1991). *In vitro,* the two variant polypeptides, PrP(Met129) and PrP(Val129), polymerised, in a nucleation-dependant manner, into fibrils following a short lag (Come & Lansbury, 1994). Kinetically, the two variants could not be distinguished, in that homogenous supersaturated solutions (i.e. above critical concentration for ploymerisation) exhibited similar lag times and rates of fibril growth. Moreover, homogenous peptide solutions could be seeded, both by homogenous seeds and by pure heterologous fibrils. In both cases, the rate of fibril growth was unaffected (Come & Lansbury, 1994). However, heterogeneous solutions

of the variant peptides exhibited longer lag times and slower growth (Figure 1.9), thereby demonstrating that unlike seeding, nucleation is sensitive to the polymorphism.

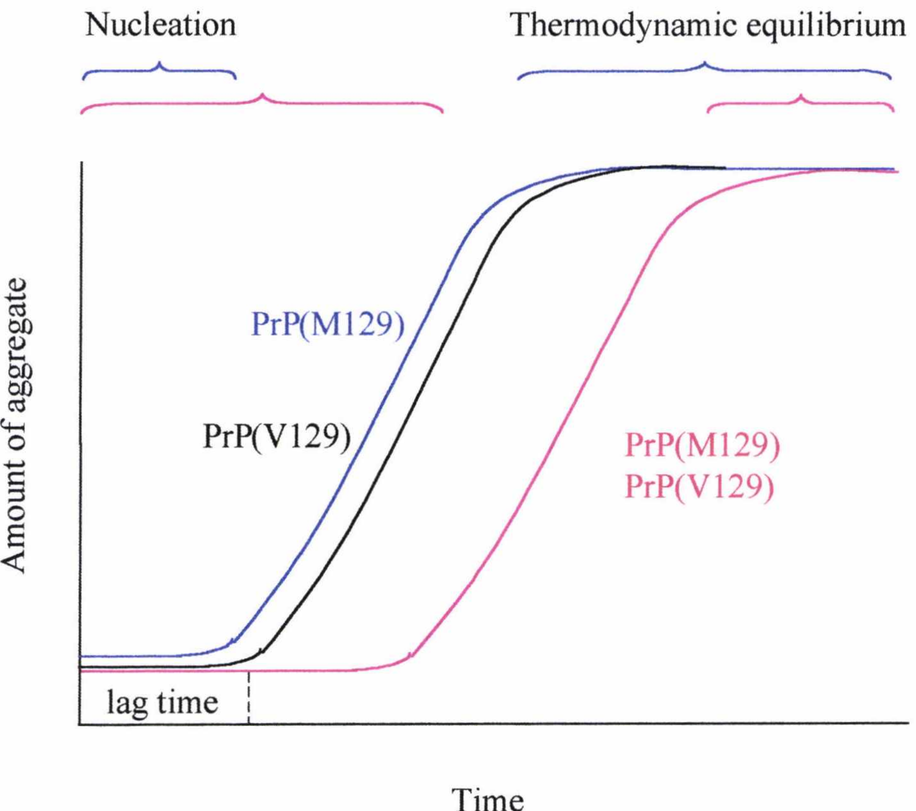


Figure 1.9 ***In vitro*** **assessment of a** ***Prnp*** **polymorphism (Come & Lansbury, 1994).** The kinetic profiles of aggregate formation are represented. In the polymerisation experiments, the same total concentration of protein was employed in each reaction (i.e. 300μM in the homogenous solutions and 150μM PrP(M129) + 150μM PrP(V129) in the heterogeneous mixture.

Remarkably, homopolymer formation was not observed in the mixed reaction and the heterogeneous solution exhibited a longer lag time (i.e. nucleation), suggesting the unfavoured production of heterogeneous nuclei (Come & Lansbury, 1994). A mixed solution of the peptides PrP(M129) and PrP(M129) also exhibited a reduced rate of polymer growth. Furthermore, dissolution studies demonstrated that heterogeneous fibrils were much less stable than homogenous filaments and therefore unfavoured thermodynamically (Come & Lansbury, 1994).

The two polymorphic variants, PrP(M129) and PrP(V129), nucleate and polymerise more slowly *in vitro*. *In vivo,* it is proposed that heterozygotes would be protected against sporadic CJD, since the formation of nuclei *de novo* is thermodynamically unfavoured. Similarly, the growth of heterogeneous amyloid is slower, thereby offering protection against amyloid propagation, in both heritable and transmitted CJD. The observation that heteropolymers are more soluble than homopolymers (i.e. dissolve more rapidly) suggests that heterogeneous amyloid would be susceptible to solubilisation and clearance. This requires that *in vivo,* amyloid formation is a reversible process. It has been demonstrated that this is the case for amyloid formation, in association with Alzheimer's disease (Maggio *et al.*, 1992).

1.6.3 Sup35p aggregation *in vitro*

It has been reported that both Sup35p [Figure 1.10] and Ure2p form highly ordered fibres *in vitro* (Glover *et al.* 1997, King *et al* 1997). Full-length Sup35p or the N-terminal residues, which constitute the PFD, form fibrils that are rich in β-sheet, resistant to degradation by proteinase K and bind Congo red.

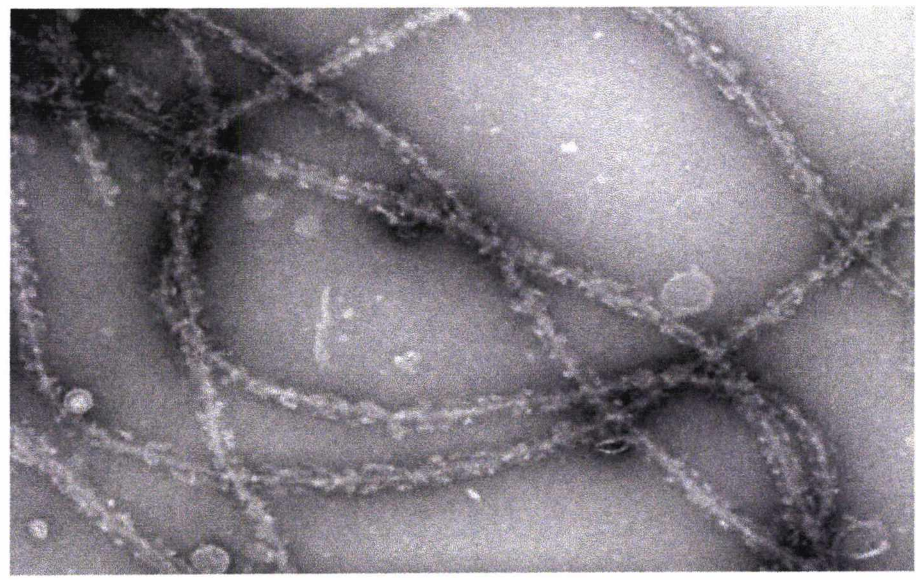


Figure 1.10 Sup35p polymerises in *vitro* to form amyloid fibrils (Glover *et al.*, 1997). As determined by electron microscopy, the central rod of Sup35p fibrils was extremely long and had an apparent diameter of 10.6 $\pm$ 1.0 nm.

The polymerisation of monomeric Sup35p *in vitro* exhibits a lag time, which is reduced by increasing monomer concentration (Glover *et al.*, 1997). Furthermore, this lag is decreased or totally eliminated upon the addition of preformed fibres (Glover *et al.*, 1997a; DePace *et al.*, 1998). These data demonstrate that *in vitro*, Sup35p polymerises in a nucleation-dependent manner (Jarrett & Lansbury, 1993). Whole-cell lysates, of *[PSI⁺]* and *[psi⁻]* strains, have been tested for their ability to promote the *in vitro* aggregation of both full-length and the PFD of Sup35p (Glover *et al.*, 1997; Paushkin *et al.*, 1997). Remarkably, only the lysates from *[PSI⁺]* strains 'seeded' aggregation, whereas *[psi⁻]* lysates failed to promote any change in the solubility of Sup35p. The nucleation-dependent polymerisation of Sup35p *in vitro* and the properties of the *[PSI⁺]* determinant, lead to the conclusion that the maintenance and propagation of the yeast prion-like element *[PSI⁺]*, was likely to be achieved by the same self-perpetuating, ordered assembly process *in vivo* (Glover *et al.*, 1997). Currently, it appears that this hypothesis has been widely accepted (Lansbury, 1997a; Paushkin *et al.*, 1997a; Derkatch *et al.*, 1998).

The cell-free fibril-formation assay has also been employed in an elegant study of *SUP35* mutations, which either reduce or eliminate *[PSI⁺]*-induced allosuppression (DePace *et al.*, 1998). *ASU* mutant proteins are poorly sequestered into Sup35p amyloid and hence mediate efficient translation termination [i.e. AntiSUppression]. *PNM* mutant proteins also elicit antisuppression, but ablate the propagation of wild-type Sup35p amyloid and the *[PSI⁺]* determinant itself, resulting in a *Psi No More* phenotype (Doel *et al.*, 1994; DePace *et al.*, 1998; see section 1.2.3 above). Generally, these mutant proteins exhibited decreased rates of seeded polymerisation and increased lag times, in unseeded reactions (DePace *et al.*, 1998). Thus, the antisuppressor properties of these mutant Sup35p appear to arise from a decreased propensity to assemble into oligomers, thereby increasing the intracellular concentration of soluble release factor and efficient translation termination. Both *ASU* and *PNM* mutant proteins have been shown to be soluble, aggregated or distributed between both states *in vivo* (DePace *et al.*, 1998; Kochneva-Pervukhova *et al.*, 1998). Undoubtedly, the continued study of Sup35p amyloid formation *in vitro*, will further the understanding of its prion-like rearrangement of conformation and how *ASU* and *PNM* mutant proteins perturb the propagation of the prion *in vivo*.

1.7 Scope of this thesis

After thirty years, the unusual genetic properties of the determinants *[PSI^{+}]* and *[URE3]* have been convincingly explained. Both are examples of protein-based inheritance in the yeast *Saccharomyces cerevisiae* (Wickner *et al.*, 1994; Tuite & Lindquist, 1996; Lindquist, 1997). The heritable phenotype reflects the three dimensional structure of a prion-like protein determinant. Most recently, the *[PSI^{+}]* determinant, Sup35p, has been shown to exhibit many biophysical properties of the scrapie prion protein, PrP. It appears that Sup35p is another member of a growing family of amyloidogenic proteins, which share little sequence homology, but all exhibit a propensity to polymerise, apparently via a common seeded mechanism, into amyloid fibres (reviewed in Kelly, 1997; Lansbury, 1997a, b). The eukaryotic organism *S. cerevisae* clearly has the potential to aid the study of amyloidogenic proteins. The yeast prion protein Sup35p has become the subject of intense research, both *in vivo* and *in vitro*. The properties of Sup35p and its interaction with endogenous factors, such as the stress protein Hsp104p, might yet serve to direct the study of amyloidogenic proteins in mammals.

An understanding of the mechanism of elimination of the yeast prions, by growth in the presence of GuHCl, was considered to be of obvious benefit. This study has challenged the accepted model for this phenomenon and has developed a novel hypothesis, which describes a putative post-translational modification that is required for yeast prion propagation *in vivo*. It is proposed that GuHCl blocks this critical modification and thereby precludes prion replication. Whilst the mammalian prion has a pathogenic 'phenotype', the determinants *[PSI^{+}]* and *[URE3]* appear to decrease the efficiency of specific cellular processes, but do not elicit a disease state. This thesis describes data that suggest that *[PSI^{+}]* is 'conserved' in yeast, since it affords a novel mechanism by which translation termination efficiency is regulated, in response to changes in environmental stress. This suggests that prions are more than just misshapen proteins and that the activity of many cellular processes might be mediated in such a manner.

Chapter II

Translation termination efficiency can be regulated in *Saccharomyces cerevisiae* by environmental stress through a prion-mediated mechanism

Chapter

II

Prion-mediated regulation of translation termination

2.1 Abstract

[PSI⁺] is a protein-based heritable phenotype of the yeast *Saccharomyces cerevisiae*, that reflects the prion-like behaviour of the endogenous Sup35p protein release factor. *[PSI⁺]* strains exhibit a marked decrease in translation termination efficiency, which permits decoding of translation termination signals and presumably, the production of abnormally extended polypeptides. We have examined whether the *[PSI⁺]*-induced expression of such an altered proteome might confer some selective growth advantage, over *[psi⁻]* strains. Although otherwise isogenic *[PSI⁺]* and *[psi⁻]* strains show no difference in growth rates under normal laboratory conditions, we demonstrate that *[PSI⁺]* strains do exhibit enhanced tolerance to heat and chemical stress, compared to *[psi⁻]* strains. Moreover, we also show that the prion-like determinant *[PSI⁺]* is able to regulate translation termination efficiency in response to environmental stress, since growth in the presence of ethanol results in a transient increase in the efficiency of translation termination and a loss of the *[PSI⁺]* phenotype. We present a model to describe the prion-mediated regulation of translation termination efficiency and discuss its implications in relation to the potential physiological the role of prions in *S.cerevisiae* and other fungi.

2.2 Introduction

It is some thirty years since the extrachromosomal determinant *[PSI⁺]* was first described in the yeast *Saccharomyces cerevisiae*, as a modifier of nonsense suppression (Cox, 1965). The *[PSI⁺]* determinant confers a dominant allosuppressor phenotype, with *[PSI⁺]* strains exhibiting a marked increase in the read-through of mutant translation termination codons (Cox *et al.*, 1988). In 1994, Wickner proposed that *[PSI⁺]* might represent an example of protein-based inheritance, whereby the heritable

element behaves like an endogenous 'prion-like' determinant (Wickner, 1994). The term prion was first used to describe the proteinaceous agent thought to be solely responsible for the family of neurodegenerative diseases, known as the transmissible spongiform encephalopathies (Griffith, 1967; Prusiner, 1982; Weissman, 1996; Horwich & Weissman, 1997).

In yeast, the *[PSI⁺]* factor is a product of the *SUP35* gene (Chernoff *et al.*, 1993; Doel *et al.*, 1994, Ter-Avanesyan *et al.*, 1994) which encodes eRF3 (Sup35p), an essential eukaryotic polypeptide release factor. Eukaryote translation termination is mediated by a soluble cytoplasmic complex, which encompasses eRF3 and at least one other factor, namely eRF1 [Sup45p] (Stansfield *et al.*, 1995a; Zhouravleva *et al.*, 1995). As well as folding into its native structure, Sup35p is believed capable of adopting a second aberrant conformation, which manifests as the prion-associated phenotype (Chernoff *et al.*, 1995a; Paushkin *et al.*, 1996; Tuite & Lindquist, 1996). In *[PSI⁺]* strains, Sup35p is present both as a soluble factor and as large intracellular aggregates, resulting from the propensity of the prion conformer to coalesce (Patino *et al.*, 1996; Paushkin *et al.*, 1996). The resulting intracellular depletion of soluble termination factors facilitates the decoding of termination signals by mutant nonsense suppressor tRNAs, or by near-cognate aminoacyl-tRNAs, giving rise to the prion-associated allosuppressor phenotype. Intriguingly, both Sup35p and the mammalian prion protein (PrP) possess a conserved N-terminal domain, comprising several imperfect glutamine-rich repeats (Oesch *et al.*, 1985; Kushnirov *et al.*, 1988). Whilst this motif is not a prerequisite for the etiology of the PrP-associated diseases (Fischer *et al.*, 1996), the N-terminal domain of Sup35p is essential for the maintenance of *[PSI⁺]* (Ter-Avanesyan *et al.*, 1994). Although the prion-inducing domain of Sup35p is not essential for translation termination or viability, it is nonetheless conserved and expressed in *S.cerevisiae* (Ter-Avanesyan *et al.*, 1993; Doel *et al.*, 1994).

Whilst no detrimental phenotype has been associated with *[PSI⁺]*, neither has a benefit been ascribed to the presence of the prion-inducing domain of Sup35p and its prion-associated phenotype. One possible scenario is that the conservation of this prion-associated domain may represent a novel means of environmental adaptation, whereby *[PSI⁺]*-induced allosuppression permits decoding of translation termination signals,

resulting in an altered pattern of gene expression and the 'profitable' generation of abnormally extended polypeptides (Lindquist, 1997). In an effort to identify such a phenotypic advantage, the stress tolerance of otherwise isogenic *[PSI⁺]* and *[psi⁻]* strains was compared. A strain bearing a nuclear allosuppressor mutation of the *SUP35* gene (*sal3-4*) was also studied, since this mutation elicits a similar translation termination deficiency as the *[PSI⁺]* element, without the associated polymerisation of eRF3. Using the *ade2-1/SUQ5* (Cox, 1965) and *lacZ*-'read-through' reporter systems (Firoozan *et al.*, 1991; Stansfield *et al.*, 1995b), it was possible to study allosuppression in both *[PSI⁺]* and *sal3-4* strains, when grown under both 'resting' and 'stressed' conditions. Our findings suggest that *[PSI⁺]* strains do exhibit a phenotypic difference to *[psi⁻]* strains, namely enhanced tolerance to physical and chemical stresses and we conclude that the prion-like behaviour of Sup35p may facilitate the adaptation of yeast to new growth environments.

2.3 Results

2.3.1 *[PSI⁺]* does not influence growth rate

The only phenotypic difference between *[PSI⁺]* and *[psi⁻]* strains so far described is the enhanced efficiency of nonsense suppression (Cox, 1965) and the elevation of stop codon readthrough in quantitative assays (Firoozan *et al.*, 1991) in *[PSI⁺]* cells. Given that >90% of the underlying Sup35p protein - an essential translation termination factor - is present as non-functional aggregates in *[PSI⁺]* strains (Patino *et al.*, 1996; Paushkin *et al.*, 1996) then one might expect *[PSI⁺]* strains to have an associated slow growth phenotype. Three *[PSI⁺]* strains were selected on the basis of their difference in genetic background and for the presence of the *ade2-1/SUQ5* reporter system (Cox, 1965). The *[psi⁻]* variant of each strain was induced by growth in the presence of 2.5mM guanidine hydrochloride, a compound which induces a *[PSI⁺]* to *[psi⁻]* reversion with near 100% efficiency (Tuite *et al.*, 1981a). The exponential growth of the three pairs of *[PSI⁺]* and *[psi⁻]* strains was compared in minimal medium at 30°C (Figure 2.1).

As demonstrated by the identical growth profiles of the *[PSI⁺]* and *[psi⁻]* variant of each of three strains, it is clear that the prion-like determinant *[PSI⁺]* does not influence the exponential propagation of yeast in typical laboratory culture conditions, with the doubling times of each pair being essentially identical.

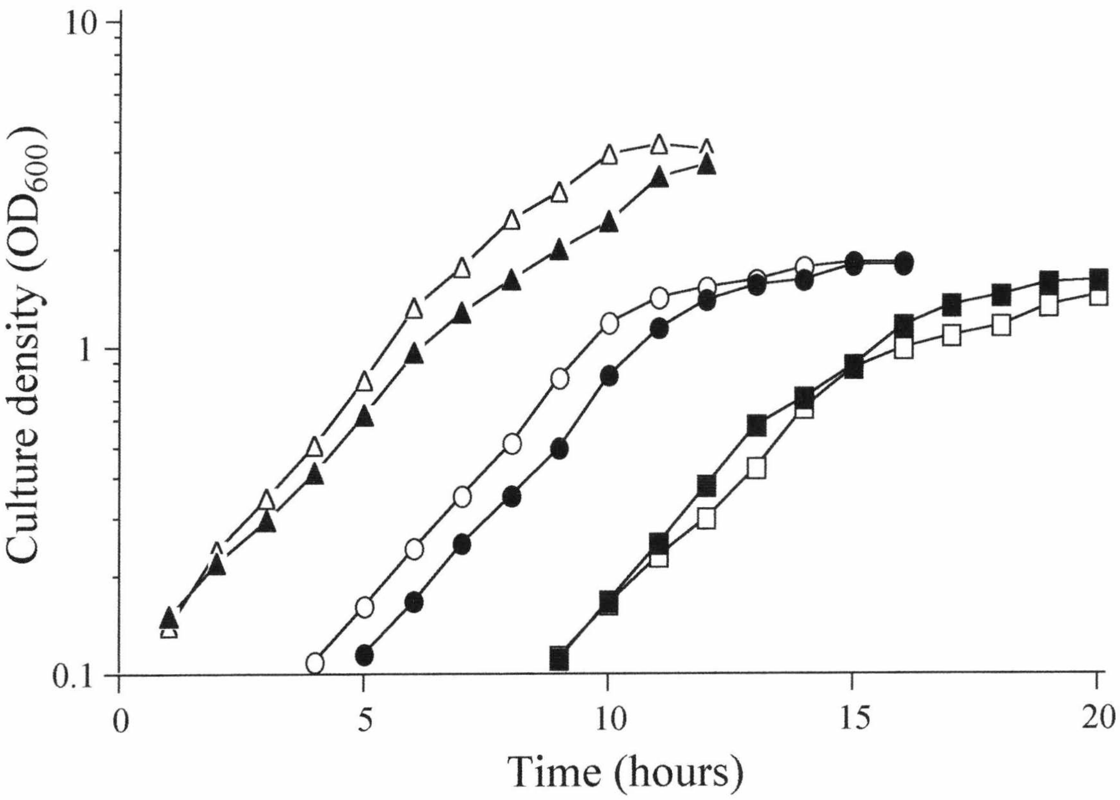


Figure 2.1 ***[PSI⁺]* and *[psi⁻]* variants of *S.cerevisiae* strains show no difference in growth under resting conditions.** The exponential growth of the *[PSI+]* (filled symbols) and *[psi-]* (empty symbols) variants of three strains was compared. Strains BSC 783/4a (●○), MT 766/12a (△▲) and BSC 772/9d (■□) were grown in minimal medium at 30°C.

2.3.2 [PSI$^+$] strains exhibit an enhanced thermotolerance

Whilst *[PSI$^+$]* does not elicit any obvious change in growth rate, it was considered that the prion-associated termination-deficiency might manifest as an activation of the cellular stress-response. The stress-response machinery acts to protect cells against the detrimental effects of changes in the extracellular environment, such as an increase in temperature or ethanol concentration. Stresses that induce the synthesis of aberrant proteins also lead to the overexpression of a particular subset of proteins, namely the heat-shock or stress proteins. For example, in yeast, the antibiotic paromomycin that stimulates mistranslation on cytoplasmic ribosomes, also induces the heat shock response (Grant *et al.*, 1989). In a termination-deficient *[PSI$^+$]* background, the production of abnormal C-terminally extended proteins might also be expected to trigger such a response. Alternatively, the presence of aggregated Sup35p itself might trigger the stress-response.

Thermotolerance assays were therefore used in order to quantify any stress-response conferred by the *[PSI$^+$]* determinant. Strains subjected to a short period of stress or experiencing a constant stimulus of the stress-response, will display an enhanced viability upon exposure to a lethal temperature, reflecting an elevation of the intracellular concentration of heat-shock proteins (Sanchez & Lindquist, 1990; Mager & Ferreira, 1993). Thermotolerance analysis of the three pairs of strains demonstrated that, for two of the three pairs, the *[PSI$^+$]* strains exhibited a significant increase in thermotolerance, in comparison to the corresponding *[psi$^-$]* variants (Figure 2.2). However, in the third strain examined (BSC 772/9d), no difference of thermotolerance was observed between the *[PSI$^+$]* and *[psi$^-$]* variants. This particular strain has a 'naturally' high degree of thermotolerance (LD_{50} of 25 min at 52°C; Figure 2.2) which we assume masks any affect *[PSI$^+$]* may have had on thermotolerance.

Given the integral relationship between *[PSI$^+$]* and Hsp104p (Chernoff *et al.*, 1995a), strain MT766/12a was employed to address the role, if any, of Hsp26p in the maintenance of *[PSI$^+$]* or the stress-induced prion-mediated regulation of translation termination efficiency. Clearly, this *HSP26*-disruptant strain can support *[PSI$^+$]*,

thereby demonstrating that Hsp26p is not essential for *[PSI⁺]* maintenance. Our data support previous studies which demonstrate that Hsp26p has little, if any, role in thermotolerance [Figure 2.2] (Tuite *et al.*, 1990). Whilst *[PSI⁺]* strains exhibited an enhanced thermotolerance, Western blot analyses failed to reveal any difference in the endogenous level of the heat shock proteins Hsp104, Hsp70 and Hsp26 between the *[PSI⁺]* and *[psi⁻]* variants, when grown at 25°C. Similarly, whilst an elevation of Hsp104p expression was detected upon heat-shock at 37°C, no significant difference was observed between *[PSI⁺]* and *[psi⁻]* strains (data not shown).

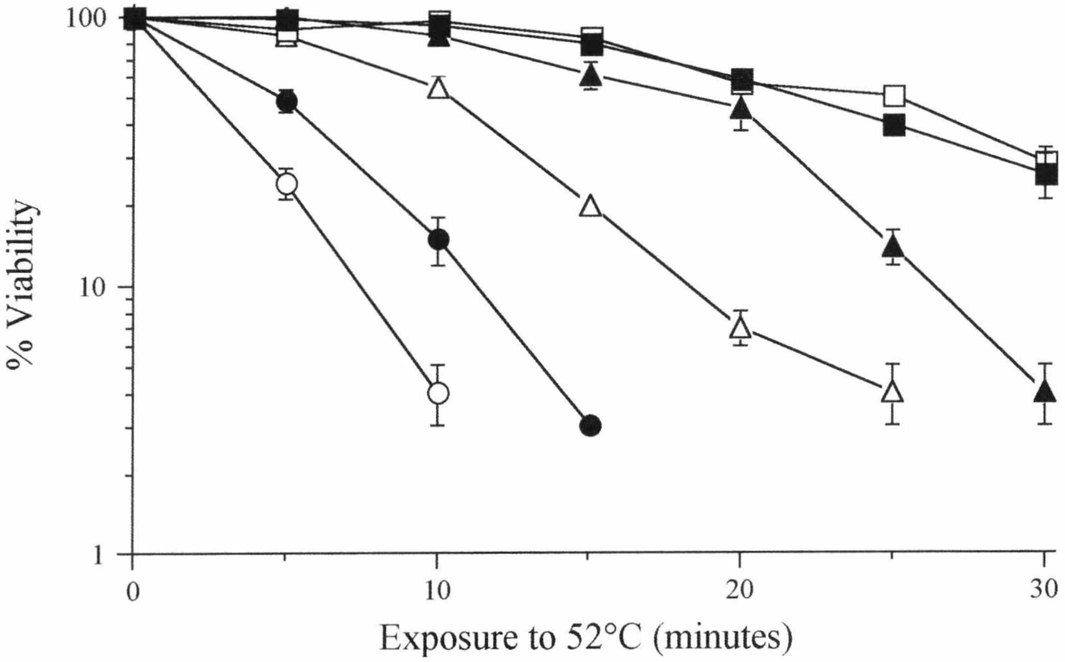


Figure 2.2 ***[PSI⁺]* strains exhibit an enhanced tolerance to thermal stress.** The viability of *[PSI⁺]* (filled symbols) and *[psi⁻]* (empty symbols) variants of three strains BSC 783/4a (●○), MT 766/12a (△▲) and BSC 772/9d (■□) was monitored, upon exposure to a lethal temperature of 52°C, following a 1 hour pre-treatment at 37°C. At regular intervals, aliquots were removed from the cultures at 52°C and put on ice. Samples were diluted and then spread in triplicate, onto solid YEPD medium to generate a viable count. Each sample gave rise to ~400 cells per plate and the error bars illustrate the variation between three viable counts per sample.

2.3.3 *[PSI⁺]* strains exhibit enhanced chemotolerance

Having demonstrated that *[PSI⁺]* enhances thermotolerance, we next asked whether *[PSI⁺]* enhanced tolerance to other forms of stress. *In vivo,* some components of the stress-response machinery do confer tolerance to more than one form of stress. For example, Hsp104p is known to be the principal agent that confers tolerance to both heat and ethanol (Sanchez & Lindquist, 1990; Sanchez *et al.*, 1992; Piper, 1995). The growth of the *[PSI⁺]* and *[psi⁻]* variants was therefore compared on gradient agar plates, which contain an increasing concentration of stress reagent in a rich growth medium. As with thermotolerance, the *[PSI⁺]* strains exhibited a greater degree of tolerance to 'chemical stress' than the *[psi⁻]* variants. The most marked enhancement of chemotolerance was observed when the strains were grown on gradients of ethanol (EtOH) (Figure 2.3). By contrast, no difference in growth was observed between the *[PSI⁺]* and *[psi⁻]* variants when grown on gradients of glycerol or salts such as sodium, potassium or magnesium chloride (data not shown). Therefore *[PSI⁺]* strains appear to show a higher degree of resistance to potential environmental abuses than the corresponding *[psi⁻]* strains.

Figure 2.3 ***[PSI⁺]* strains exhibit an enhanced tolerance to ethanol.** The stress tolerance of the *[PSI⁺]* and *[psi⁻]* variants of each of the three test strains was compared, by growth upon solid YEPD medium, containing a gradient of 0-10% v/v ethanol. Strain suspensions were normalised with respect to cell density and equal volumes (i.e. numbers) of cells were spotted either onto solid ¼YEPD medium (control) or onto a plate containing a gradient of 0-10% Ethanol. Plates were then incubated at 30°C for 5 days.

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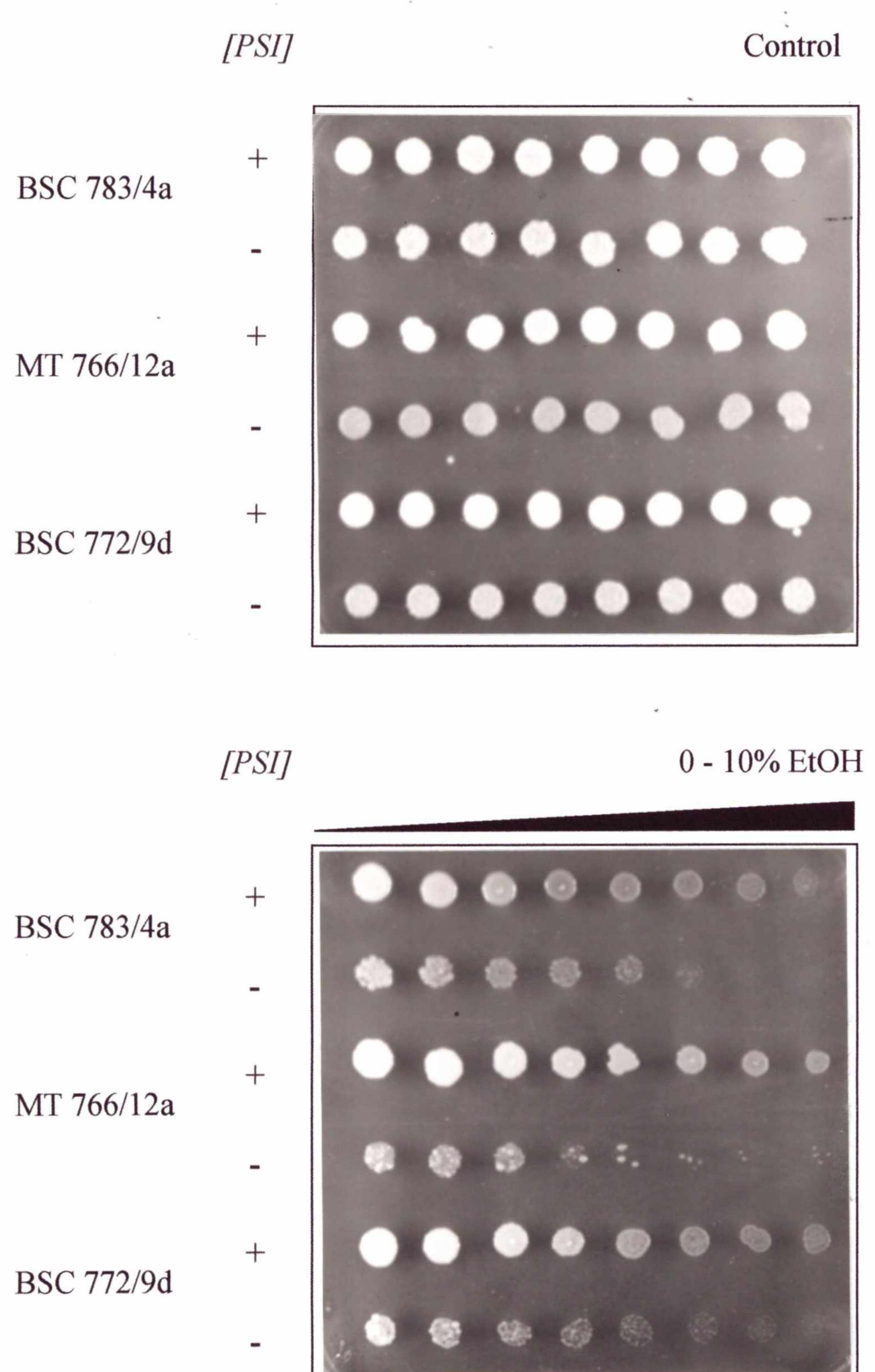
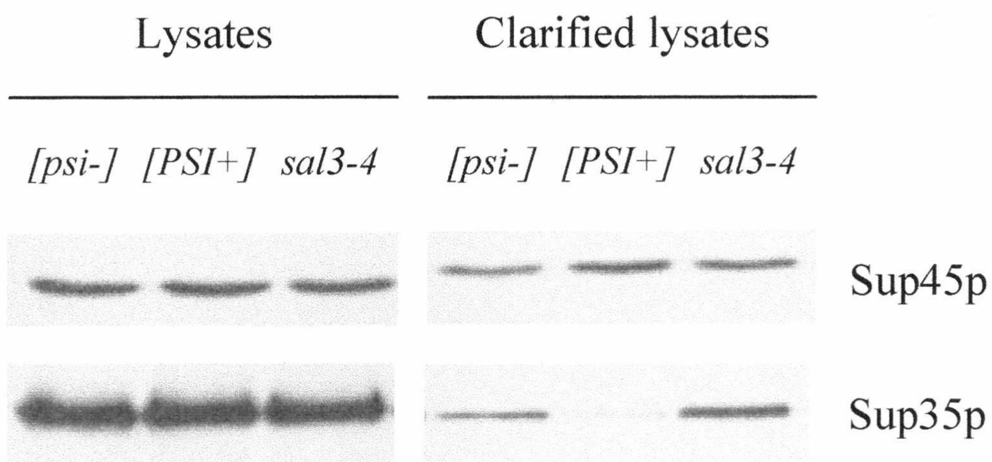


Figure 2.3

2.3.4 Allosuppression enhances stress tolerance

To determine how the *[PSI⁺]*-determinant elicits an enhanced stress tolerance, the thermo- and chemotolerant properties of a [*PSI⁺*] strain and a [*psi⁻*] strain bearing the *sal3-4* mutation were compared. The *sal3-4* mutation lies in the *SUP35* gene (Doel *et al.*, 1994) presumably within the functional C-terminal domain of the encoded eRF3 thereby leading to a termination defect not through Sup35p aggregation, but from biochemical malfunction. Western blot analysis of the *sal3-4* mutant strain, using polyclonal antiserum raised against Sup35p, confirmed that unlike the *[PSI⁺]* strains under examination, the *sal3-4* allele encodes primarily soluble Sup35p.



<u>Figure 2.4</u> **The *sal3-4* mutant encodes a soluble part-functional component of the eukaryote release factor.** The sedimentation properties of the two eukaryotic release factors, Sup35p and Sup45p, were determined in the *[PSI⁺]* and *[psi⁻]* variant of strain BSC 783/4c and in a mutant strain, bearing the nuclear allosuppressor allele, *sal3-4*. Proteins were detected by Western blot analyses, of whole-cell lysates and the soluble fraction of total cell extracts, following the clarification of lysates by centrifugation.

The *[PSI⁺]* strain BSC783/4c and the *sal3-4* allosuppressor mutant elicited a similar degree of termination codon read-through (Table 2.1) and thus are presumed to elicit the same degree of production of C-terminally extended polypeptides. Like the *[PSI⁺]* strains, the *sal3-4* mutant did not exhibit an impairment of exponential growth (data not shown). The *sal3-4* mutant also showed enhanced thermotolerance (Figure 2.5a) and ethanol tolerance (Figure 2.5b), thereby demonstrating that it is a deficiency of translation termination that is the principal trigger for an elevated, intracellular stress response in *[PSI⁺]* strains, rather than the presence of Sup35p aggregates.

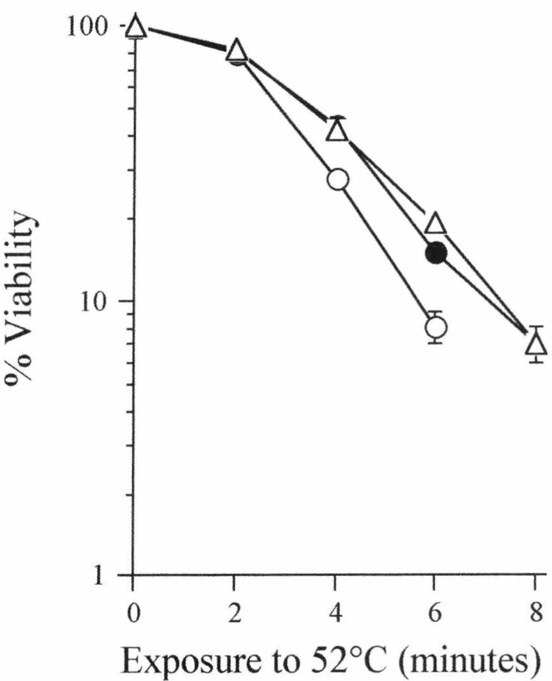


Figure 2. 5a The prion-like determinant *[PSI⁺]* and the nuclear allosuppressor mutation *sal3-4* both elicit enhanced thermotolerance. The thermotolerance of the *[PSI⁺]* (●) and *[psi⁻]* (○) variants of BSC 783/4c was compared to that of a *[psi⁻]* variant of BSC 783/4c, that bears the nuclear allosuppressor allele *sal3-4* (△). Cultures were exposed to a lethal temperature (52°C), following a 1hour pre-treatment at 37°C. At regular intervals, culture aliquots were removed from 52°C and put on ice. Samples were then diluted and spread in triplicate, onto solid YEPD medium to generate a viable count. Each sample gave rise to ~400 cells per plate and the error bars represent the variation between three viable counts per sample.

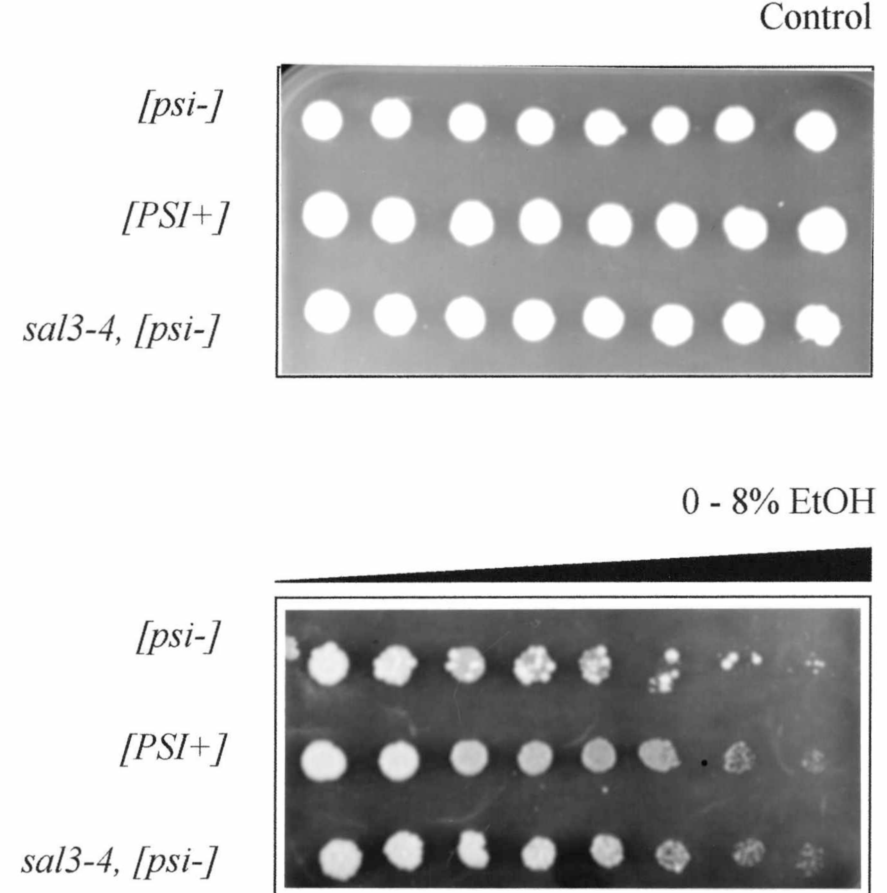
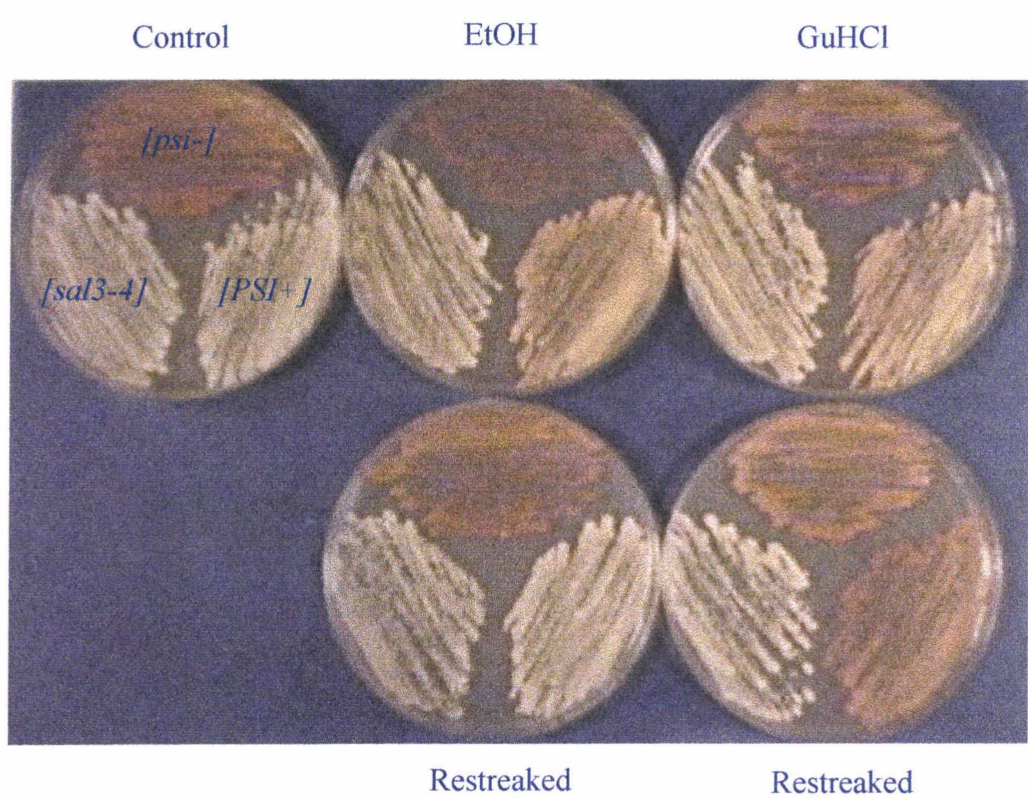


Figure 2. 5b The prion-like determinant *[PSI$^+$]* and the nuclear allosuppressor mutation *sal3-4* both enhance ethanol tolerance. The ethanol tolerance of the BSC 783/4c variants *[PSI$^+$]*, *[psi$^-$]* and *sal3-4, [psi$^-$]* was also compared by studying their growth on solid rich medium supplemented with ethanol. Strain suspensions were normalised with respect to cell density and equal volumes (i.e. numbers) of cells were spotted either onto solid ¼YEPD medium (control) or onto a plate containing a gradient of 0-8% Ethanol. Plates were incubated for 5 days at 30°C.

2.3.5 *[PSI$^+$]* strains exhibit antisuppression when stressed

Intriguingly, *[PSI$^+$]* strains exhibited a transient loss of their allosuppressor phenotype, when grown on certain 'chemical' media.



<u>Figure 2.6</u> **Stress induces a reversible antisuppression in *[PSI$^+$]* strains, but not in strains bearing the nuclear allosuppressor mutation *sal3-4*.** <u>Top row</u> - The variants of strain BSC 783/4c were grown on control ¼YEPD medium (left) and medium supplemented with either 5% ethanol (middle) or 2.5mM guanidine hydrochloride (right). <u>Each Plate</u> - Strain BSC 783/4c, clockwise from top *[psi$^-$], [PSI$^+$] and [psi$^-$] sal3-4*. <u>Bottom row</u> - Variants were then re-streaked from stress-inducing media (top row) onto control ¼YEPD medium. By virtue of the *ade2-1/SUQ5* genetic markers, variants that exhibit a translation termination deficiency (i.e. nonsense suppression) develop as white colonies, whereas strains efficient for translation termination accumulate a red pigment. Plates were incubated for 5 days at 30°C.

This stress-induced 'antisuppression' was not simply 'curing' of the *[PSI$^+$]* prion, (as is induced by guanidine hydrochloride, Tuite *et al.*, 1981a) since the stressed *[PSI$^+$]* strains reverted to an allosuppressor phenotype upon transfer to chemical-free media (Figure 2.6). Growth in the presence of the stress-inducing agent did not result in the heritable loss of the *[PSI$^+$]* determinant, despite the transient loss of the *[PSI$^+$]* phenotype. The degree of stress-induced antisuppression varied between the various *[PSI$^+$]* strains, but antisuppression was readily invoked by growth in the presence of ethanol.

	% Read-through		
	[psi$^-$]	*[PSI$^+$]*	*[psi$^-$] sal3-4*
0% Ethanol	4.7 $\pm$ 1.0	23.8 $\pm$ 5.7	18.5 $\pm$ 3.8
7% Ethanol	3.9 $\pm$ 0.8	7.7 $\pm$ 1.5	15.6 $\pm$ 3.0

Table 2.1 Quantification of termination efficiency in strains bearing the *[PSI$^+$]* and *sal3-4* allosuppressor determinants, upon growth in a stress-inducing medium. The level of termination signal read-through was measured in strain BSC 783/4c, using a β-galactosidase reporter system (described by Stansfield *et al.* 1995b). Read-through of termination codons was quantified using the plasmid pUKC817 (which carries the *lacZ* gene that bears a pre-mature termination codon) and expressed as a proportion of control β-galactosidase levels, measured in transformants carrying the control plasmid pUKC815 (which carries the wild-type *lacZ* gene).

It is noteworthy that strain MT766/12a, also exhibited a transient antisuppressor phenotype when grown at 37°C (unpublished data). By contrast, a strain bearing the nuclear allosuppressor mutation *sal3-4* failed to exhibit antisuppression in response to stress (Table 2.1, Figure 2.6). Thus, strains bearing the prion-like determinant *[PSI$^+$]* exhibit a reversible decrease in termination codon read-through (i.e. antisuppression) when grown under stress, which unlike growth on guanidine hydrochloride, does not result from the elimination of the *[PSI$^+$]* determinant.

2.4 Discussion

The two yeast prion elements so far described, namely *[PSI⁺]* and *[URE3]*, may both serve as regulators of different cellular physiological processes, namely translation termination and nitrogen catabolism respectively (Cox, 1994; Wickner & Masison, 1996). Since this realisation, there has been considerable speculation as to the much wider role of prions in biological systems (Patino *et al.*, 1996; Tuite & Lindquist, 1996; Wickner & Masison, 1996; Lindquist, 1997). Indeed, this notion appears to be supported by the recent description of a prion-like element, which confers heterokaryon incompatibility, in the filamentous fungus *Podospora anserina* (Coustou *et al.*, 1997). If prion elements and their associated heritable traits are prevalent throughout different species, they should presumably confer some benefit to the organism carrying them. By studying the growth of *[PSI⁺]* and *[psi⁻]* yeast strains under adverse environmental conditions, we have demonstrated that the yeast prion *[PSI⁺]* does indeed confer a beneficial phenotypic difference, namely that of enhanced tolerance to environmental stress.

Both the prion-like determinant *[PSI⁺]* and the nuclear allosuppressor mutation *sal3-4* are derived from the *SUP35* gene and confer a partial translation termination defect to yeast (Table 2.1). In this study, we have shown that the *sal3-4* mutation elicits an increase in translation termination codon read-through, similar to that conferred by the *[PSI⁺]* determinant and that the *sal3-4* allosuppressor mutation results in the expression of a soluble, but partially non-functional polypeptide release factor. This is different to *[PSI⁺]*-induced allosuppression, that arises from the prion-like propensity of a conformer of Sup35p to aggregate, which results in the intracellular depletion of functional soluble termination factor (Paushkin *et al.*, 1996, 1997b; reviewed in Tuite & Lindquist, 1996). Both prion- and nuclear mutation-induced allosuppression produces an enhancement of thermo- and chemotolerance. Plausibly, the read-through of translation termination codons elicits an enhancement of stress-tolerance via a constitutive stress response, which is invoked by the presence of proteins that have misfolded C-terminal extensions. This notion has also been applied to account for the evolution of a $tRNA^{Ser}$ of *Candida albicans*, which inserts serine at the leucine-

encoding codon CUG (Santos *et al.*, 1996). Transformation of *S.cerevisiae*, with a gene encoding this recoded tRNA, generates an alternative 'mistranslated' proteome that also results in an enhancement of stress tolerance.

Rather than increasing stress tolerance by triggering a constitutive stress response, it might be suggested that *[PSI⁺]*-induced allosuppression (and indeed the same would be true of the *sal3-4* mutation) permits the production of one or more novel protein(s) which bear an extra C-terminal domain. The modified biochemical properties of such elongated polypeptides might directly enhance stress tolerance; for example if these extended polypeptides stabilised the cell membrane. Whilst *[PSI⁺]* leads to an increased tolerance of *S.cerevisiae* to adverse environmental conditions, *[PSI⁺]* is not a regulator of the stress response. The potentially beneficial phenotype of enhanced stress tolerance is a consequence of *[PSI⁺]*-induced allosuppression. Whereas strains bearing the *sal3-4* allosuppressor mutation do not exhibit a significant change in termination efficiency, in response to increased environmental stress, the prion-like element *[PSI⁺]* mediates a reversible, stress-induced increase in translation termination efficiency (Figure 2.6 and Table 2.1). Paradoxically, the elevated intracellular stress response which results from the prion-induced production of extended polypeptides (and manifests as an enhancement in thermotolerance), is selected against in an adverse environment since the generation of misfolded proteins is disfavoured by the prion-mediated decrease in the extent of translation termination codon read-through.

Deletion analysis has revealed that whilst the N-terminal domain of Sup35p is essential for the maintenance of *[PSI⁺]*, it is not essential for cell viability and thus appears to play no direct functional role in translation termination (Ter-Avanesyan *et al.*, 1993, 1994). However, this glutamine-rich domain of Sup35p has been conserved in *S.cerevisiae*, either as an indirect result of the *[PSI⁺]* phenotype or because of some as yet unidentified function. Whilst *[PSI⁺]* has recently been described as a 'laboratory-confined disease' of *S.cerevisiae* (Chernoff *et al.*, 1998), we have demonstrated that the prion element *[PSI⁺]* does not influence exponential growth, but does actually confer a beneficial phenotype to yeast, namely enhanced stress tolerance. Intriguingly, there is evidence to suggest that Sup35p does serve a role in eukaryotes, other than that of a polypeptide release factor. For example, Sup35p has been identified as a putative cell

cycle factor of *S.cerevisiae* (Kikuchi *et al.*, 1988) and recently the *Drosophila* homologue of yeast Sup35p has been shown to mediate meiotic spindle assembly (Basu *et al.*, 1998).

In *S.cerevisiae, [PSI$^+$]* represents a novel mechanism for the inheritance of a regulated translation termination deficiency. Clearly, the lethal consequences of a nonsense mutation in an essential gene would be overcome in an allosuppressor background. *[PSI$^+$]* is an omnipotent allosuppressor determinant, in that it enhances nonsense suppression of all three termination codons (Firoozan *et al.*, 1991), thus *[PSI$^+$]* would be expected to offer protection against most potentially lethal nonsense mutations. One such example has been described in which a strain exhibits a *[PSI$^+$]*-dependence, since it bears a nonsense mutation within the essential gene *HSF1*, which encodes the heat shock transcription factor (Lindquist & Kim, 1996). Alternatively, *[PSI$^+$]* might permit the regulated expression of abnormally extended polypeptides, which results from the extended decoding of open reading frames (ORF) by by-passing ORF-defining termination codons. Genome analysis has revealed the existence of several potentially 'read-through-regulated' genes (Lindquist, 1997) but as a caution, it should be noted that translation termination is not only regulated by the interaction of release factors with termination codons. In particular, the efficiency of nonsense suppression and hence the expression of any abnormally extended polypeptide, will also be governed by the presence and properties of endogenous nonsense suppressor tRNA and by the 'context' of the stop signal within the mRNA (Fearon *et al.*, 1994; Bonetti *et al.*, 1995).

Our data suggest that *[PSI$^+$]* does not promote the alternative expression of genes whose products are absolutely required for growth under adverse conditions. Indeed, the reversible antisuppression induced by stress may serve to reduce termination codon read-through, thereby preventing the harmful production of extended polypeptides. The *FLO8* gene has been identified as a likely 'regulated' template in the strain S288C (Liu *et al.*, 1996; Lindquist, 1997). This gene product is essential for filamentous growth and yet this strain bears an apparently 'internal' termination codon. Whilst *[PSI$^+$]*-induced allosuppression would be expected to elicit the expression of 'full-length' Flo8p and permit filamentous growth of S288C, we predict that the environmental conditions that normally induce filamentous growth would also invoke the transient

shift of the *[PSI⁺]* strain to an antisuppressor state, thereby reducing nonsense suppression of termination codons and precluding pseudohyphae formation.

As proposed by Lindquist (1997), the interplay between stress-proteins (Hsp104p) and prions does provide a plausible molecular mechanism for a cell to respond to its environment with a heritable change in phenotype. We would expand this concept to suggest that the yeast prion *[PSI⁺]* actually represents a mechanism for the regulation of translation termination efficiency in response to changes in environmental conditions, rather than a simple switching between a *[PRION⁺]* and *[prion⁻]* heritable phenotype. Cells possess specific mechanisms for tolerating adverse environmental conditions and for the recovery and elimination of stress-denatured proteins. One such mechanism in *S.cerevisiae* involves the action of the stress protein Hsp104p, which is known to be important for protecting yeast against the detrimental effects of heat and alcohol (Sanchez & Lindquist, 1990; Sanchez *et al.*, 1992; reviewed in Piper, 1995). Moreover, Hsp104p is a prerequisite for the maintenance and propagation of *[PSI⁺]* (Chernoff *et al.*, 1995a). We propose that the stress-mediated regulation of translation termination efficiency in *[PSI⁺]* strains arises from the increased levels of stress-proteins, which mediates the partial regeneration of functional Sup35p and hence a transient increase in translation termination efficiency. Thermal and ethanol stress also induce the elevated expression of Hsp104p and Ssa1p [Hsp70p] (reviewed in Mager & Ferreira, 1993; Piper, 1995), which is known to result in the loss of *[PSI⁺]*-induced allosuppression (Chernoff *et al.*, 1995a; Paushkin *et al.*, 1996). We postulate that Hsp104p is the primary factor in *[PSI⁺]*-meditated regulation of translation termination efficiency, in response to environmental stress. Clearly, not all stress-induced proteins have a role in this phenomenon, for example Hsp26p (this study). As a regulatory mechanism, the interplay between the stress proteins and the prion protein is such that the *[PSI⁺]* determinant is not eliminated from the cell. Upon removal of the environmental stress and the subsequent decrease in the intracellular level of stress proteins, Sup35p is sequestered by residual 'seeds' into large intracellular polymers, resulting in the restoration of the *[PSI⁺]* allosuppressor phenotype (Figure 2.7).

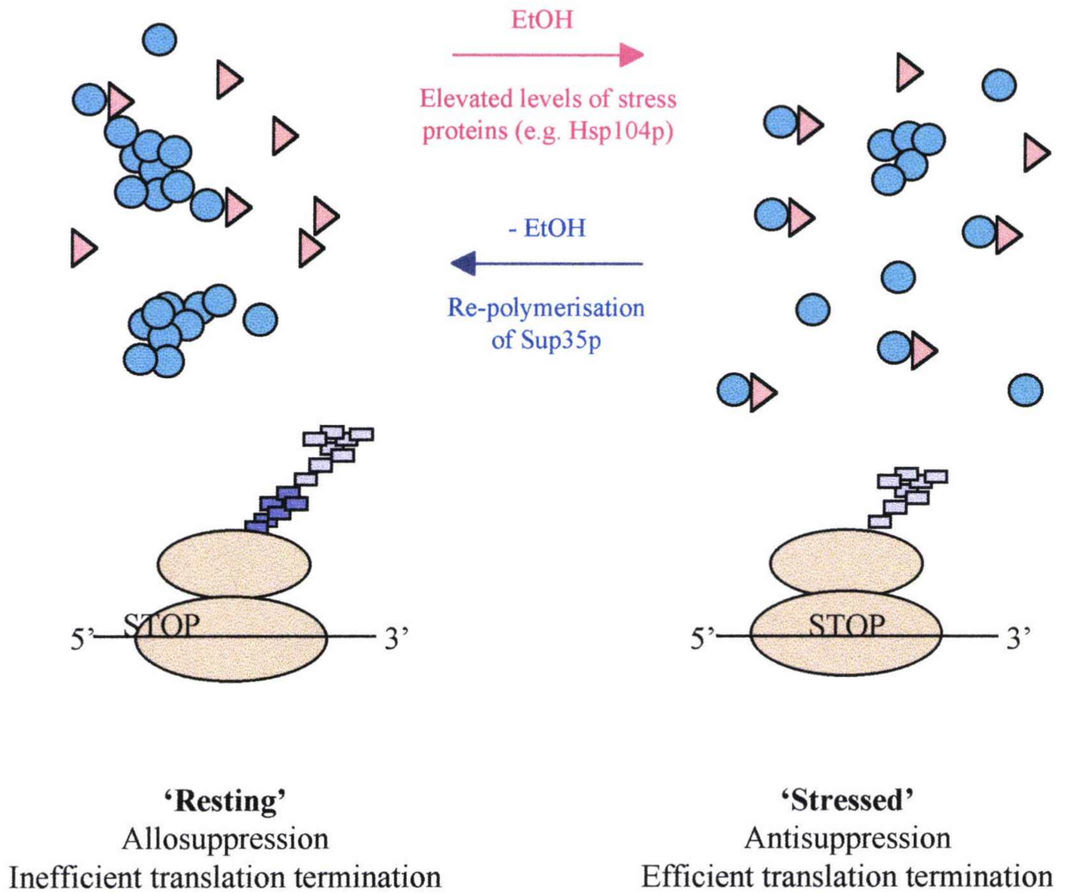


Figure 2.7 **A model for the prion-mediated regulation of translation termination.** In a *[PSI⁺]* background, efficient translation termination is prevented by the intracellular polymerisation of Sup35p and a depletion of functional eRF3 (Sup35p) [○] - eRF1 (Sup45p) [△] termination factor complex. An increase in environmental stress, such as an increase in ethanol concentration, transiently elevates the production of stress proteins (e.g. Hsp104p), which mediates the partial resolubilisation of Sup35p aggregates. The emergence of soluble Sup35p elicits an increase in translation termination efficiency. Upon restoration of the cell to a normal environment, the expression of stress proteins subsides and allosuppression is restored by the sequestration of Sup35p into 'aggregates' by residual *[PSI⁺]* seeds.

2.5 Materials and methods

2.5.1 Strains

Three *[PSI⁺]* strains were selected on the basis of their difference in genetic background and for the presence of two specific genetic elements: (1) the ochre suppressor serine-inserting tRNA encoded by the *SUQ5* gene and (2) the *ade2-1* mutation, which permit a direct visualisation of allosuppression status by colony-colour (Cox, 1965). Strains exhibiting efficient translation termination give rise to red colonies, whereas those strains displaying an allosuppressor phenotype (i.e. termination inefficient) grow white. The three strains were rendered prion-deficient (i.e. *[psi⁻]*) on the basis of a stable white to red colony-colour change, that occurs upon growth on media containing 2.5mM guanidine hydrochloride (Tuite *et al.*, 1981a).

The genotypes of the four strains used in these studies were:
BSC783/4a: *SUQ5, ade2-1, ura3-1, his3-11, his3-15, leu2-3, leu2-112, MATa*
BSC783/4c: *SUQ5, ade2-1, ura3-1, his3-11, his3-15, leu2-3, leu2-112, MATa*
BSC772/9d: *SUQ5, ade2-1, ura3-1, his4-166, leu2-2, lys1-1, can1-100, MATa*
MT766/12a: *SUQ5, ade2-1, ura3-1, hsp26::HIS3, MATa*

2.5.2 Growth media

Yeast strains were grown either in liquid YEPD complete medium (2% w/v glucose, 1% w/v Bacto-peptone, 1% w/v yeast extract) or defined minimal medium (0.67% Difco defined minimal medium without amino acids, 2% glucose) supplemented with the appropriate amino acids and cofactors (20mg/L except adenine which was added at 200mg/L). For colony-colour enhancement, yeast strains were grown on ¼YEPD solid medium (4% w/v glucose, 1% w/v Bacto-peptone, 0.25% w/v yeast extract, 2% w/v agar). The reduced content of yeast extract in ¼YEPD ensures the maximum accumulation of the chromogenic adenine biosynthetic precursor. For studies using

ethanol-supplemented media, strains were grown in flasks, sealed with rubber bungs, to reduce evaporation of the alcohol from the medium.

2.5.3 Sedimentation analysis of polypeptide release factors

Yeast strains were grown to an OD_{600} of 0.5, harvested and lysed with glass beads at 4°C, in buffer (25mM Tris-HCl pH 8, 150mM NaCl, 5mM EDTA, 5mM PMSF, Complete protease inhibitor mix [Boehringer Mannheim, 1 tablet per 5ml buffer]). Lysates were then clarified by centrifugation for 10 minutes at 13000 x g. and stored on ice. Samples of lysate and clarified lysate were boiled for 10 minutes, after the addition of 5 x SDS-PAGE sample buffer (2.5mL glycerol, 1.25mL 20% v/v SDS, 0.25mL 2-mercaptoethanol, 1.25mL 0.5M Tris-HCl pH 6.8). Samples were cooled on ice and then spun for 5 minutes at 13000 x g. Protein samples were analysed using 10% SDS-polyacrylamide gels and electrophoretically transferred to nitrocellulose for Western blot analysis, employing polyclonal sera raised against yeast Sup35p and Sup45p expressed in *E.coli*, as previously described (Stansfield *et al.*, 1995a).

2.5.4 Thermotolerance assays

Strains were grown at 25°C to mid-exponential phase in YEPD (OD_{600} 0.4) and then transferred to 37°C. Following a one hour pre-treatment, strains were diluted to a density of approximately 3.5 x 10^3 cells per ml. Strains were then transferred to a 52°C shaking water bath, whereupon aliquots were removed at regular intervals and stored on ice. 150μL aliquots (approximately 500 cells) were plated in triplicate onto solid YEPD agar and viable counts were determined after 5 days growth at 30°C.

2.5.5 Chemotolerance assays

A fresh colony of each strain was taken up from a ¼YEPD plate, resuspended in 500μl sterile water and diluted to approximately 1 x 10^6 cells per ml. 8 x 2μl strain suspension were spotted onto a chemical gradient plate. Each plate comprised 50mL ¼YEPD agar supplemented with a stress reagent, which was poured 24 hour prior to use. The stress agar was allowed to set with the plate tilted, so that there was no stress agar at one end of the plate. The plates were used following the addition of 50ml of 'top' ¼YEPD agar and as soon as the top agar had set. Following the spotting of the strain suspensions, all gradient plates were sealed to prevent evaporation of stress-inducing agents and then incubated for 5 days at 30°C.

2.5.6 Quantifying allosuppression levels *in vivo*

Yeast strains were transformed with one of the two 'read-through' vectors pUKC815/ pUKC817 (Stansfield *et al.*, 1995b). The single-copy control vector pUKC815 carries the *lacZ* gene under the control of the constitutive *PGK*-promoter. The read-through vector pUKC817 has an in-frame premature ochre (TAA) termination signal. Strains were grown in appropriate minimal media to an OD_{600} of 0.45, whereupon the cells from 10ml culture aliquots were harvested and stored at -20°C. Strains were grown in the presence of 7% v/v ethanol for at least 24 hours, to ensure a true reflection of read-through levels (i.e. stress exposure was prolonged to permit the 'turn over' of any residual β-galactosidase produced during growth in normal media). The degree of allosuppression was determined as a function of the β-galactosidase levels of the strains bearing the read-through vectors.

The cell pellets from the culture aliquots were resuspended in 600ul buffer Z (60mM Na_2HPO_4, 40mM NaH_2PO_4, 10mM KCl, 1mM $MgSO_4$, pH 7.0) supplemented with EDTA (5mM final), 2-ME (50mM final) and PMSF (5mM final). Cells were lysed at 4°C by vortexing 3 times for 30 seconds (with 30 second intervals on ice) in the

presence of an equal volume of glass beads. β-galactosidase and Bradford assays were then performed on lysates clarified by centrifugation (10min, 13000 x g, 4°C), as described (Stansfield *et al.*, 1995b). 50ml clarified lysate was diluted with 750μl Z and warmed to 37°C. The assay was initiated by the addition of 160μl *ortho*-nitrophenylgalactoside (4mg/ml, aqueous) and conducted at 37°C. Following 20 minutes incubation, the reaction was quenched by the addition of 480μl di-sodium carbonate (1M, aqueous). The absorbance at 420nm was recorded and an extinction co-efficient of 0.0045 ml. $nmol^{-1}$ was used to calculate the amount of *ortho*-nitrophenol (ONP) produced. β-galactosidase activity was determined as nmol ONP produced min^{-1}, mg^{-1} of protein in the assay and expressed as a percentage of the control activity (i.e. that of clarified lysates from strains bearing the control vector pUKC815).

$$\beta\text{-galactosidase activity} = \frac{\text{(ONP produced) nmol}}{\text{(Assay time) 20 min} \times \text{(Total protein) mg}}$$

$$\text{(ONP produced) nmol} = \frac{\text{A420}}{0.0045\ \text{ml. nmol}^{-1}} \times \text{(Assay volume) ml}$$

$$\text{(Total protein) mg} = {}^{*}\text{[Protein] mg.ml}^{-1} \times \text{(Assay volume) ml}$$

* As determined by Bradford assay, using a commercial test kit (Biorad, UK)

Chapter III

Guanidine hydrochloride blocks a critical step in the propagation of the prion-like determinant *[PSI^+]* of *Saccharomyces cerevisiae*

Chapter

Elimination of [PSI^+] by guanidine hydrochloride

3.1 Abstract

The cytoplasmic heritable determinant *[PSI^+]* of the yeast *Saccharomyces cerevisiae*, reflects the prion-like properties of the chromosome-encoded protein Sup35p. This protein is known to be an essential eukaryote polypeptide release factor, namely eRF3. In a *[PSI^+]* background, the prion conformer of Sup35p forms large oligomers which results in the intracellular depletion of functional release factor and hence inefficient translation termination. We have investigated the process by which the *[PSI^+]* determinant can be efficiently eliminated from strains, by growth in the presence of the protein denaturant guanidine hydrochloride (GuHCl). Strains are 'cured' of *[PSI^+]* by millimolar concentrations of GuHCl, well below that normally required for protein denaturation. Here we show that the elimination of the *[PSI^+]* determinant is neither derived from the direct dissolution of self-replicating *[PSI^+]* seeds by GuHCl, nor from the action of an ancillary factor (i.e. Hsp104p) which catalyses the refolding of the prion conformer of Sup35p to its native structure. Whilst GuHCl does elicit a stress response, the elimination of *[PSI^+]* is not enhanced by stress and furthermore, exhibits an absolute requirement for continued exponential growth. We propose that GuHCl inhibits a critical event in the propagation of the prion-conformer and demonstrate that the kinetics of curing fit a model whereby the heritable *[PSI^+]* element is diluted from a culture, following the total inhibition of prion replication by GuHCl.

3.2 Introduction

Recent biochemical evidence (Patino *et al.*, 1996; Paushkin *et al.*, 1996; Glover *et al.*, 1997; King *et al.*, 1997; Paushkin *et al.*, 1997a) has supported the hypothesis that the *[PSI⁺]* phenotype of *Saccharomyces cerevisiae* reflects the prion-like properties of the *SUP35* gene product (Cox, 1994; Wickner, 1994). The essential chromosome-encoded protein Sup35p is known to be one of two eukaryote polypeptide release factors, namely eRF3 (Stansfield *et al.*, 1995a, Zhouravleva *et al.*, 1995). Sup35p associates with Sup45p (eRF1) *in vivo,* to mediate translation termination (Stansfield *et al.*, 1995a). *In vitro,* Sup35p forms highly ordered fibers, whose appearance resembles that of fibrils formed by other amyloidogenic polypeptides (Glover *et al.*, 1997; King *et al.*, 1997). In a *[PSI⁺]* background, most Sup35p exists as large aggregates, possibly reflecting the propensity of this protein to form amyloid fibres *in vivo* (Patino *et al.*, 1996; Paushkin *et al.*, 1996). This property manifests as an allosuppressor phenotype (i.e. translation termination inefficient) in *[PSI⁺]* cells, presumably because the cell is depleted of functional termination factors (Paushkin *et al.*, 1996, 1997b; reviewed in Tuite & Lindquist, 1996). In normal growth conditions, *[PSI⁺]* strains are metastable, with a relatively low frequency of reversion to *[psi⁻]* (Lund & Cox, 1981; Tuite *et al.*, 1981a). However, Tuite *et al.* (1981a) demonstrated that growth in media containing very low concentrations (1-5mM) of guanidine hydrochloride (GuHCl) converted yeast cells, with up to 100% efficiency, from *[PSI⁺]* to *[psi⁻]*. Similar observations were made for *[URE3]*, a second prion determinant of *S.cerevisiae* (Wickner, 1994). Other reagents including methanol, ethylene glycol and hypertonic conditions, have been reported to exhibit curing properties; however, none of these cure with the near-total efficiency of GuHCl (Singh *et al.*, 1979; Tuite *et al.*, 1981a).

Two hypotheses have been proposed, to account for the curing properties of GuHCl. Firstly, the elimination of the prion might arise directly, from the ability of GuHCl to denature proteins. However, the concentrations of GuHCl effective in curing *[PSI⁺]* are in the millimolar range, rather than the molar range typically required to promote the denaturation of proteins *in vitro* (Tuite *et al.*, 1981a). Alternatively, GuHCl may actually promote the expression of an ancillary factor, namely the stress protein

Hsp104p, which indirectly results in the reactivation of Sup35p and consequently the loss of the prion (Patino & Lindquist, *cited in* Chernoff *et al.*, 1995a). Unlike other heat-shock proteins, Hsp104p does not act to protect proteins against stress (i.e. heat denaturation) rather, Hsp104p actively promotes the recovery of stress-denatured aggregated proteins by facilitating their refolding back into functional, native conformations (Parsell *et al.*, 1994a; Glover & Lindquist, 1998). Overexpression of Hsp104p might lead to the total refolding of Sup35p from the aberrant prion conformation to its native structure, thereby mediating prion-loss. In order to test both hypotheses, we have examined the kinetics of prion elimination upon growth in the presence of GuHCl and assessed the influence of stress upon the curing process. Furthermore, we have studied the effects of GuHCl upon the sedimentation properties of Sup35p in order to probe the relationship between aggregation and the *[PSI⁺]* phenotype.

3.3 Results

3.3.1 Exponential growth is a prerequisite for curing by GuHCl

To study the profile of *[PSI⁺]* elimination from *S.cerevisiae* by GuHCl, the *[PSI⁺]* variant of strain BSC783/4a was grown at 30°C in rich YEPD medium, supplemented with 3mM GuHCl. Two identical cultures were developed, one of which was allowed to enter stationary phase, following a short period of exponential growth, encompassing approximately four doubling times (i.e. ~10 hours). The other culture was grown in identical medium, but was diluted into fresh YEPD + GuHCl as required (i.e. when the OD_{600} reached 0.8, a density of approximately 6 x 10^6 cells.ml^{-1}), to ensure continued exponential growth. During the course of the experiment, culture aliquots were removed at hourly intervals and the cells harvested. The cells were washed with sterile water, diluted and aliquots were spread onto ¼YEPD solid medium (typically 100 - 300 colony forming units per plate), in order to determine the ratio of *[PSI⁺]* and *[psi⁻]* cells in the culture. The composition of the culture was determined on the basis of a white/red colony ratio, by virtue of the *ade2-1/SUQ5* marker system (Cox, 1965).

Sectored colonies were scored according to the simplest model of segregation (i.e. a half-white, half-red colony was scored as two individual cells, one being *[PSI⁺]* and one *[psi⁻]*). As seen in Figure 3.1, GuHCl was only able to elicit prion loss from a *[PSI⁺]* culture that had sustained continued exponential growth. Despite progressing through at least four cycles of cell division, *[PSI⁺]* was not eliminated from the stationary phase culture; thereby demonstrating that cell division is a prerequisite for curing by GuHCl. Intriguingly, the profile of prion-elimination exhibited a significant lag, corresponding to approximately four generations, before the gradual loss of *[PSI⁺]* cells from the culture.

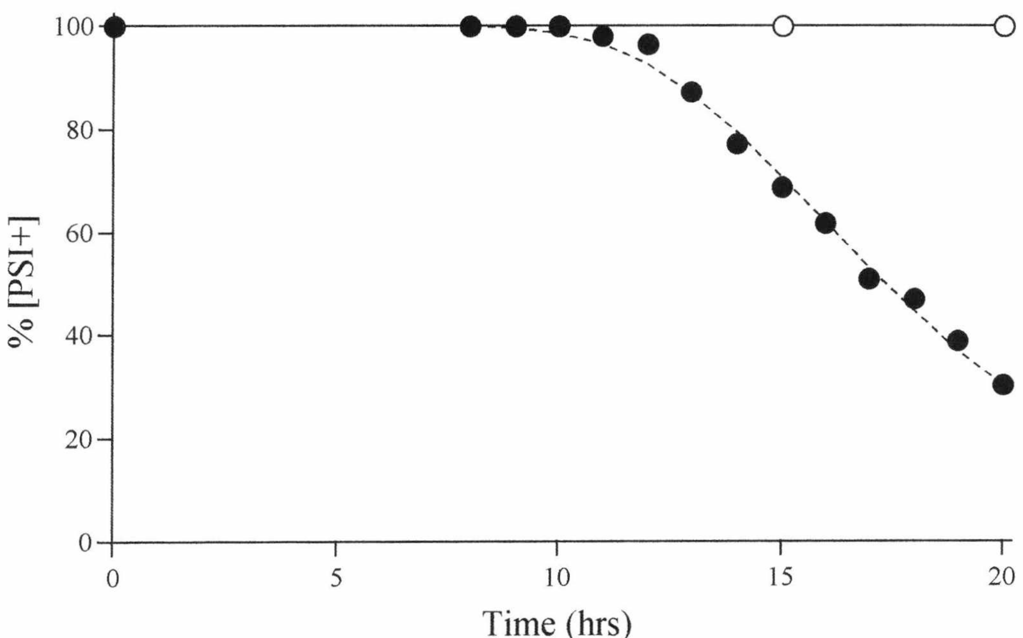


Figure 3.1 Elimination of the *[PSI⁺]* determinant by GuHCl requires cell division. Two identical cultures of the *[PSI⁺]* strain BSC783/4a, were grown at 30°C in YEPD supplemented with 3mM GuHCl. One culture was maintained in exponential growth (●), whilst the other was allowed to enter stationary phase (○), after 10 hours (approximately four generations) of exponential growth. The *[PSI⁺]* fraction of the cultures were determined, as a function of time, on the basis of the red/white colony ratio of culture aliquots, grown on solid ¼YEPD medium. Each data point represents an average of three culture samples. The dashed line represents the best fit to a simple segregational model, with variables pre-existing prion seeds and generation time (see Chapter 3.5.5).

3.3.2 Curing by GuHCl and the dissolution of Sup35p aggregates

The prion-associated phenotype *[PSI⁺]* is believed to arise from the intracellular polymerization of Sup35p and a corresponding depletion of soluble translation termination factor. To complement our analysis of the effects of GuHCl upon a culture of *[PSI⁺]* cells, we studied the sedimentation of Sup35p in cells, following exponential and stationary phase growth, in the presence of GuHCl. Western blot analysis of non-fractionated whole-cell lysates (i.e. in the complete absence of centrifugation), demonstrated that the total intracellular level of Sup35p did not vary in response to GuHCl (Figure 3.2). Therefore, GuHCl does not alter the expression of the *SUP35* gene *per se*.

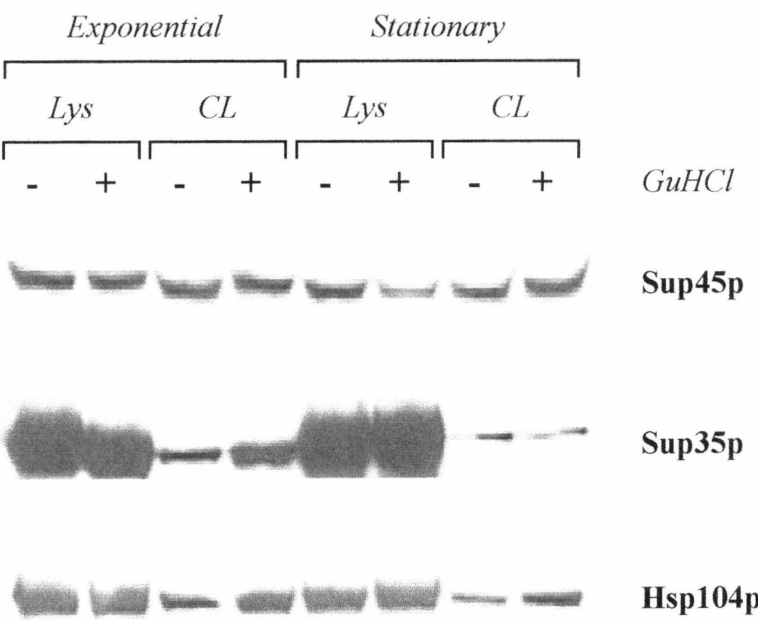


Figure 3.2 Effects of GuHCl upon the sedimentation properties of Sup35p. The *[PSI⁺]* strain BSC783/4a was grown for 25 hours (~10 generations) at 30°C in YEPD, in the absence or presence of 3mM GuHCl. Cultures were either maintained in exponential phase or allowed to enter stationary phase, after 10 hours exponential growth. Whole-cell lysates (Lys) were clarified by centrifugation (CL) and then analyzed by Western blot.

Surprisingly, a large fraction of the total intracellular Sup35p was always found to sediment, upon centrifugation of whole-cell lysates (Figure 3.2). Again, any effect that GuHCl had upon Sup35p, or upon a factor that facilitates curing, exhibited an absolute requirement for cell division. *[PSI⁺]* cells grown exponentially, in the presence of GuHCl, exhibited an increased soluble fraction of total Sup35p (Figure 3.2). In contrast, the soluble fraction of total Sup35p was no different in stationary phase cultures, which were grown in the presence or absence of GuHCl. The data demonstrate that GuHCl does not, either directly or indirectly, promote the dissolution of 'sedimentable' Sup35p to the soluble form. Whilst a decrease in the sedimenting-fraction of total Sup35p need not reflect a loss of self-replicating prion seeds (see Chapter 3.4), the data show that GuHCl does not promote the dissolution of any pre-existing seeds, since *[PSI⁺]* is maintained in the stationary phase culture containing the curing agent.

3.3.3 GuHCl invokes a stress response in yeast

It has been proposed that GuHCl facilitates the loss of *[PSI⁺]* indirectly, by enhancing the expression of an ancillary factor, namely Hsp104p (Patino & Lindquist *cited in* Chernoff *et al.*, 1995a). Hsp104p is the principal protein of yeast that confers thermal and ethanol tolerance (Sanchez & Lindquist, 1990; Sanchez *et al.*, 1992; Lindquist & Kim, 1996). As well as being essential and sufficient for survival at elevated temperatures, a specific level of Hsp104p is required for the maintenance of *[PSI⁺]*. A *[PSI⁺]* culture converts to *[psi⁻]* in an excess or absence of Hsp104p (Chernoff *et al.*, 1995a). Plausibly, GuHCl could elicit the overexpression of Hsp104p, which itself mediates the production of native Sup35p by catalysing the refolding of the aberrant prion conformer (Parsell *et al.*, 1994a; Chernoff *et al.*, 1995a).

To investigate the effect of GuHCl upon the expression of Hsp104p, we studied the acquired thermotolerance of a *[PSI⁺]* strain following a non-lethal exposure to 3% v/v ethanol, 3mM GuHCl (a concentration that cures *[PSI⁺]*) and a sudden temperature shift, from 25°C to 37°C. Strains that are subjected to a mild stress pre-treatment invoke a transient expression of heat-shock proteins (primarily Hsp104p) and exhibit an

enhanced survival upon subsequent exposure to a lethal temperature (Sanchez & Lindquist, 1990). Whilst a pre-treatment at 37°C increased the thermotolerance of strain BSC783/4a (LD_{50} of 15min at 52°C, compared to control LD_{50} of 5min, Figure 3.3a), it was observed that treatment with GuHCl actually resulted in a decreased survival upon immediate transfer to a lethal temperature of 52°C. This effect was similar to that seen for ethanol, which actually sensitized strain BSC783/4a to heat and precluded the measurement of any survival at 52°C (data not shown). Cells become hypersensitive to thermal stress following the disruption of cell membranes by alcohol (Lloyd *et al.*, 1993; reviewed in Piper, 1995), suggesting that GuHCl may exert a similar effect. By washing the cells to remove any ethanol or GuHCl, following a pre-treatment, it was possible to demonstrate that GuHCl might enhance thermotolerance (Figure 3.3b).

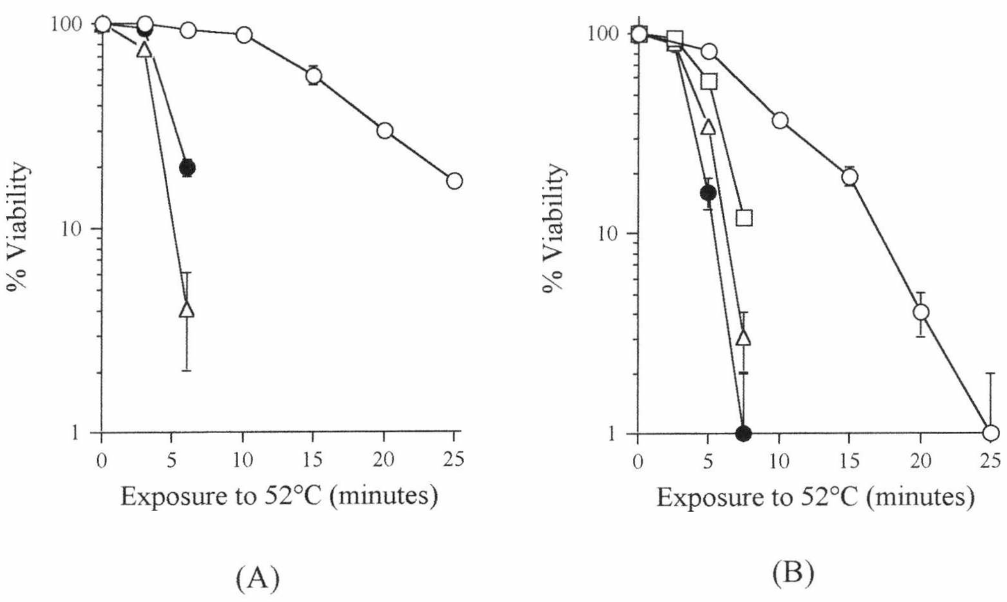


Figure 3.3 GuHCl enhances the thermotolerance of a *[PSI⁺]* strain. Cultures were grown at 25°C (●) or pre-treated for 1 hour either at 37°C (○), or in the presence of 3mM GuHCl (△) or 3% v/v EtOH (□). The survival of BSC783/4a *[PSI⁺]* at 52°C was monitored, immediately after the pre-treatment **(A)** or following a wash with sterile water **(B)**. At regular intervals, aliquots were removed from the cultures at 52°C and put on ice. Samples were diluted and then spread in triplicate, onto solid YEPD medium to generate a viable count. Each sample gave rise to ~400 cells per plate and the error bars illustrate the variation between three viable counts per sample.

By equating acquired thermotolerance with Hsp104p expression, it was shown that the stress response of strain BSC783/4a was greater to the presence of 3% v/v ethanol, than to 3mM GuHCl. However, the effects of ethanol and GuHCl were very modest compared with that observed for a thermal pre-treatment (Figure 3.3b). Furthermore, Western Blot analysis failed to detect any increase in the level of Hsp104p, following a one hour exposure to 3mM GuHCl or 3% v/v EtOH, although an enhanced production of Hsp104p was detected following a 37°C pre-treatment (data not shown).

3.3.4 Stress does not enhance curing by GuHCl

To further assess the influence of stress and Hsp104p expression upon curing, we studied the loss of the *[PSI⁺]* determinant from strain BSC783/4a, when grown in media containing 3mM GuHCl, with and without 3% v/v ethanol (Figure 3.4). The presence of 3mM GuHCl had a modest effect upon the growth of the strain in YEPD media, increasing the doubling-time by approximately 10% to 2.5 hours. When grown in YEPD supplemented with both GuHCl and ethanol, the doubling time of the strain was increased approximately two-fold, to 5.5 hours (data not shown). As demonstrated by the above thermotolerance data (Figure 3.3b), the presence of 3% v/v EtOH would be expected to elicit a greater stress-response, than that induced by 3mM GuHCl.

Whilst the addition of ethanol clearly increased the stress upon the curing culture, as reflected by an increased doubling time, any corresponding induction of heat shock protein(s) did not enhance the rate of prion elimination (Figure 3.4). Moreover, the time taken to eliminate the *[PSI⁺]* determinant was greatly increased, by the presence of ethanol. It is noteworthy that no curing was observed in an identical culture of BSC783/4a grown in YEPD media, supplemented with ethanol alone (data not shown). This highlighted the exceptional efficiency of GuHCl as a curing agent and demonstrated that *per se*, stress is a relatively inefficient curing agent (Tuite *et al.*, 1981a; Chernoff *et al.*, 1995a,b).

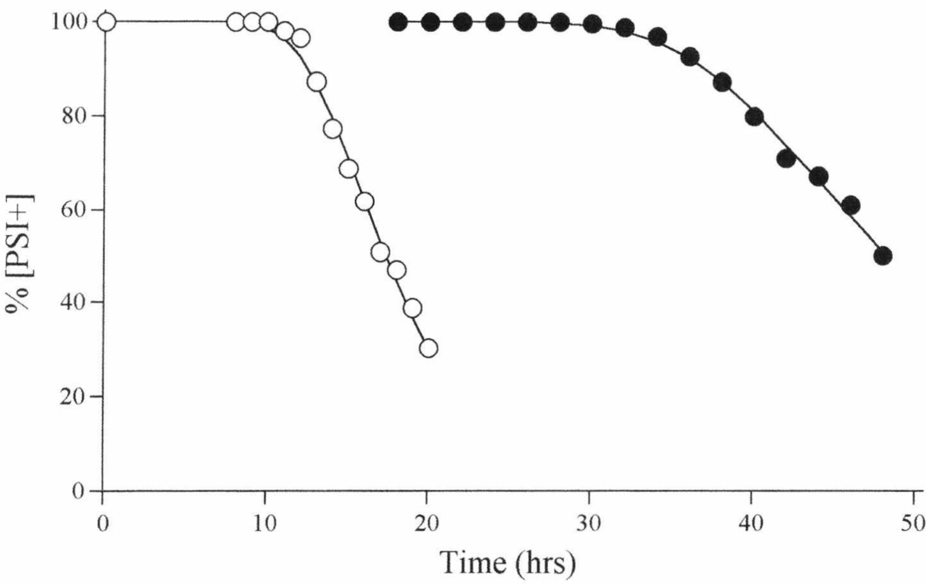


Figure 3.4 Stress-response does not enhance the rate of prion elimination by GuHCl. BSC783/4a *[PSI⁺]* was grown in exponential phase, at 30°C in YEPD containing 3mM GuHCl (○) and further supplemented with 3% v/v EtOH (●). The *[PSI⁺]* fraction of the cultures was determined, as a function of time, on the basis of the red/white colony ratio of culture samples. Each data point represents an average of three culture samples. The lines represents the best fit to a simple segregational model, with variables pre-existing prion seeds and generation time (see section 3.5.5 below).

3.3.5 GuHCl ablates propagation of the prion determinant

We propose that the profile of GuHCl-induced curing (Figure 3.1) and the absolute requirement of cell division, can best be explained by the hypothesis that GuHCl blocks a critical step in the replication of the prion conformer and that pre-existing prion seeds are 'diluted' in cells during sustained exponential growth. Our hypothesis predicts that a *[PSI⁺]* culture, grown in the presence of GuHCl, would display a segregational lag, before the emergence of prion-free cells (Figure 3.1). During this lag, the average number of prion seeds within each cell is approximately halved every doubling-time, following the random segregation of any prion seeds between parent and daughter cell. The size of the segregational lag would be dependent upon the average number of pre-existing seeds, within a *[PSI⁺]* cell. This 'segregational' model also predicts that it is possible to achieve the stable recovery of *[PSI⁺]* cells at any stage during curing,

although as time goes by the fraction of these becomes too few to detect. To test this segregational hypothesis, two aliquots were withdrawn from a culture that had been growing for 12.5 hours (five generations), in the presence of in GuHCl. The aliquots were either inoculated into fresh media containing GuHCl, or 'rescued' into YEPD media alone. As seen previously (Figure 3.1), the *[PSI⁺]* determinant was lost in an exponential manner, upon continued growth in the presence of GuHCl (Figure 3.5). However, in agreement with our hypothesis, the 'rescued' culture aliquot that was developed in the absence of GuHCl, exhibited a stable fraction of *[PSI⁺]* cells (Figure 3.5).

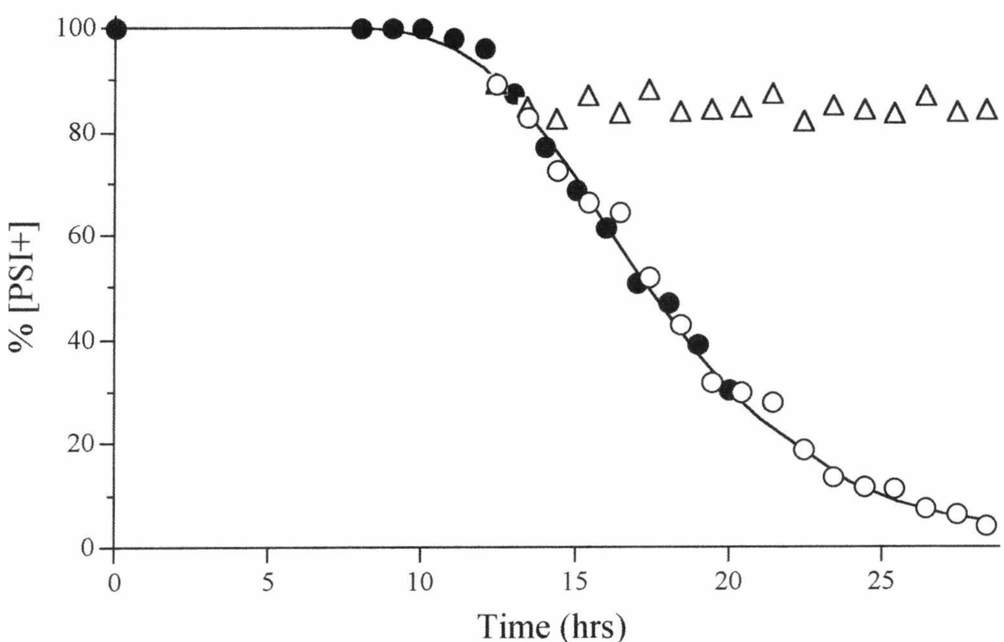


Figure 3.5 The propagation of *[PSI⁺]* is stable upon removal of cells from GuHCl. After 12.5 hours exponential growth, in the presence of 3mM GuHCl (●), two culture aliquots of BSC783/4a were transferred to fresh medium containing 3mM GuHCl (O) or to YEPD medium alone (Δ). The cultures were then allowed to continue exponential growth. The *[PSI⁺]* fraction of the cultures was determined, as a function of time, on the basis of the red/white colony ratio of culture samples. Each data point represents an average of three culture samples. The line overlaying the data for growth in the presence of GuHCl, represents the best fit to a simple segregational model, with variables pre-existing prion seeds and generation time (see section 3.5.5 below).

Whilst the addition of 3% v/v EtOH did not reduce the time taken to eliminate the *[PSI⁺]* determinant (Figure 3.4), the data did support our segregational model. First, the presence of EtOH would be expected to accelerate curing, if the stress-induced expression of an ancillary factor were the means by which GuHCl induces the loss of the *[PSI⁺]* determinant. This is not observed. Second, the GuHCl-induced curing profiles of the two cultures (in the presence or absence of EtOH) are identical, when the culture composition is analyzed with respect to the number of generations (Figure 3.6, c.f. Figure 3.4).

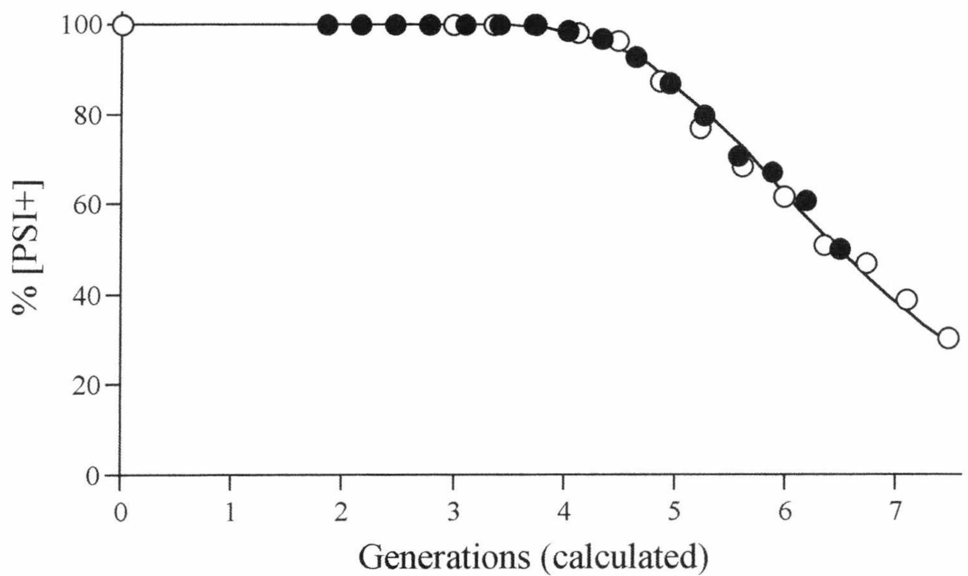


Figure 3.6 Curing fits to a simple random segregation model. The data of Figure 3.4 are re-plotted with respect to generations, as calculated by the model (see section 3.5.5 below) and adjusted to account for a brief cessation of growth, during which the cells adapted to the presence of 3% v/v EtOH. BSC783/4a *[PSI⁺]* was grown in exponential phase, at 30°C in YEPD containing 3mM GuHCl (○) and further supplemented with 3% v/v EtOH (●). The curve is a theoretical plot, based upon the segregational hypothesis, where the calculated number of pre-existing prion seeds within a cell is 62. The calculated generation times for both sets of data from the model were within 10% of the experimentally determined generation times.

The elimination of *[PSI⁺]* from a culture, growing in YEPD medium supplemented with both GuHCl and EtOH, takes much longer since the doubling time of the culture is increased. This in turn increases the time taken to 'segregate' pre-existing seeds, between parent and daughter cells, thereby making the dilution (curing) of *[PSI⁺]* a

slower process. The curve that fits the data points in Figure 3.6 is a theoretical curve, based upon our hypothesis that the replication of *[PSI⁺]* seeds is totally inhibited by GuHCl and that any pre-existing seeds then segregate, at random, between parent and daughter cells until *[psi⁻]* (i.e. seed-free) cells emerge. The curve permits a calculation of the average number of prion seeds per cell, at the time of addition of GuHCl, which is 62 $\pm$ 10.

3.4 Discussion

The *[PSI⁺]* phenotype of *S.cerevisiae* reflects the prion-like behaviour of the host-encoded protein Sup35p (eRF3). Sup35p does exhibit several properties of the mammalian prion protein, PrP (reviewed in Tuite & Lindquist, 1996; Kushnirov & Ter-Avanesyan, 1998). The prion-like properties of Sup35p can be eliminated by growth in the presence of millimolar concentrations of GuHCl (Tuite *et al.,* 1981a). This chaotropic salt is widely employed as a protein denaturant and indeed has even been shown to result in the loss of PrP-infectivity when employed at a concentration >3.5 M (Caughey *et al.,* 1995; Kocisko *et al.,* 1994, 1996). The data presented in this study do not support a mechanism of *[PSI⁺]* curing whereby GuHCl either directly, or indirectly, elicits the dissolution of any pre-existing Sup35p polymer or prion-like seeds. Furthermore, we have shown that stress does not enhance curing, either in the presence or absence of GuHCl. If the induction of the stress protein Hsp104p were the underlying factor in *[PSI⁺]* elimination, then curing might be expected to be accelerated in stationary phase cultures or those grown in the presence of ethanol. Whilst such conditions have been reported to invoke the enhanced production of Hsp104p (Sanchez *et al.*, 1992; Lindquist & Kim, 1996), we have demonstrated that they have little or no effect upon the observed rate of curing by GuHCl.

Curing by GuHCl exhibits an absolute requirement for growth and a segregational lag, which we propose reflects the particulate inheritance of *[PSI⁺]* and its subsequent dilution from cells grown in the presence of GuHCl. Our data, under all conditions tested, fits extremely well to a random segregation model for curing, whereby there are a number of self-replicating particles within a *[PSI⁺]* cell which are randomly

segregated between mother and daughter cells, following the total inhibition of their propagation by GuHCl. Whilst the average number of prion-seeds per cell decreases by half with each generation, even late in the curing curve there will be a significant number of cells with more than one prion-seed per cell. Hence, the shape of the curve for the random segregation model is therefore much shallower than a directed segregation model and shows a longer, shallower gradient than a model in which prion-seeds are evenly (i.e. non-randomly) segregated.

From our data, it is possible to calculate the number of self-replicating seeds within a *[PSI$^+$]* cell, which for strain BSC783/4a was approximately sixty (Figure 3.6; see Chapter 3.5.5). A remarkably similar number has been deduced previously, for two different *[PSI$^+$]* strains and by a completely different experimental approach (McCready *et al.*, 1977). As a provocative alternative to the prion hypothesis, the particulate pattern of *[PSI$^+$]* inheritance could be construed as evidence that *[PSI$^+$]* is in fact an autonomous nucleic acid, for example a virus or a plasmid. GuHCl is known to be a reversible inhibitor of poliovirus RNA replication at millimolar concentrations (Barton & Flanegan, 1997), it eliminates the penicillinase-encoding plasmid of *Staphylococcus aureus* (Juliani *et al.*, 1975) and it efficiently induces non-chromosomal respiratory-deficient petites of *S.cerevisiae* with extremely high efficiency (Juliani *et al.*, 1973). However, unlike curing of *[PSI$^+$]*, growth is not a prerequisite for petite-induction in yeast (Juliani *et al.*, 1973). Furthermore, despite the identification of many plasmids and viruses of yeast (reviewed in Wickner *et al.*, 1996), no extrachromosomal nucleic acid has ever been linked to *[PSI$^+$]* (Young & Cox, 1972; Tuite *et al.*, 1982).

Perhaps the most compelling evidence that *[PSI$^+$]* is a protein-only phenomenon is the requirement of a specific level of Hsp104p for *[PSI$^+$]* maintenance (Chernoff *et al.*, 1995a; Paushkin *et al.*, 1996). Originally, it was proposed that Hsp104p permits a conformational transition state in wild-type Sup35p, that facilitates its folding into a prion conformation (Chernoff *et al.*, 1995a; Lindquist, 1997). This equates to a 'breathing template' model proposed for replication of the mammalian prion, in which partial denaturation of the prion conformer is required, to permit its acting as a template for the corruption of native protein (Kocisko *et al.*, 1994; Caughey *et al.*, 1995). However, the role of Hsp104p as an essential catalyst of the native to prion-like

structural change has been disputed, following the apparent *in vitro* production of the Sup35p prion conformer in the absence of Hsp104p (Glover *et al.*, 1997; King *et al.*, 1997). Recently, it has been proposed that Hsp104p functions solely as a 'disaggregase' – Hsp104p is required to break-up Sup35p polymers in a *[PSI⁺]* strain, thereby promoting the continued inheritance of *[PSI⁺]* seeds to all mitotic (and meiotic) progeny (Paushkin *et al.*, 1996; DePace *et al.*, 1998; reviewed in Kushnirov & Ter-Avanesyan, 1998). This mechanism for generating new prion seeds has been termed secondary nucleation (Orgel, 1996). Ironically, in fulfilling its normal cellular role as a 'disaggregase', Hsp104p may actually ensure the propagation of *[PSI⁺]* in *S.cerevisiae.*

Since Hsp104p is a key factor in the replication of *[PSI⁺]*, it is plausible that GuHCl effects its activity directly, thereby resulting in the inhibition of prion replication. GuHCl could disrupt the action of Hsp104p, regardless of whether Hsp104p functions as a disaggregase to facilitate secondary nucleation, or by promoting the partial unfolding of the prion-like conformer of Sup35p, an event that may or may not be required for prion propagation. Hsp104p bears two nucleotide-binding sites, the second of which is essential for the assembly of Hsp104p into a functional homohexameric complex (Parsell *et al.*, 1991, 1994b). Mutations within these ATP-binding sites have a profound effect upon chaperone function, in terms of acquired thermotolerance (Parsell *et al.*, 1991), recovery of stress-denatured protein (Parsell *et al.*, 1994a) and most intriguingly, in the maintenance of *[PSI⁺]*. Expression of a mutant Hsp104p with both ATP-binding sites inactivated, converted *[PSI⁺]* cells to *[psi⁻]* (Chernoff *et al.*, 1995a; Patino *et al.*, 1996). This double mutation of the *HSP104* gene is a dominant P*si No More (PNM)* mutation (McCready *et al.*, 1977), in that it causes the loss of *[PSI⁺]* even in the presence of the chromosomal *HSP104* gene (Chernoff *et al.*, 1995a). The dominant effect of the double mutant is thought to arise from the oligomeric nature of functional Hsp104p. The co-expression of wild-type and mutant protein presumably gives rise to heterogenous hexamers, which are defective for nucleotide hydrolysis and hence activity (Chernoff *et al.* 1995a).

It is plausible that GuHCl has an adverse effect upon the ATPase activity of Hsp104p, resulting in a loss of function and ultimately, the loss of the prion. Glover & Lindquist (1998) demonstrated that the ATPase activity of Hsp104p is extremely sensitive to low

concentrations of GuHCl *in vitro,* although Hsp104p did not exhibit the same sensitivity to urea. Similarly, the transcriptional activity of T7 RNA Polymerase is enhanced nearly 2-fold in the presence of 50mM GuHCl and yet only exhibits denaturation and a loss of activity in the presence of urea (Das & Dasgupta, 1998). Whilst millimolar concentrations of GuHCl may not be sufficient to promote denaturation of proteins, it is clear that such levels can have a profound effect upon protein activity. As an alternative, GuHCl could actually act upon Sup35p itself. The low concentration of GuHCl could result in the stabilisation of the native Sup35p or the prion conformer itself, thereby precluding a partial denaturation event that might be required for prion replication. Such a mechanism of action has been proposed for the anion Congo red, which inhibits the corruption of native PrP to the prion conformer (Capsi *et al.,* 1998).

Whilst our data has not identified the GuHCl-sensitive step in the replication of the prion conformer of Sup35p, it has prompted us to reconsider the relationship between Sup35p aggregation and *[PSI⁺]*. Our analysis revealed that the majority of Sup35p sediments upon centrifugation of a whole-cell lysate (Figure 3.1), irrespective of the *[PSI⁺]*-status of the culture. This demonstrates the existence of at least two forms of Sup35p in a *[psi⁻]* background; a soluble fraction, which we presume to mediate translation termination and a fraction that sediments in association with 'cell debris'. We interpret this as evidence of a cytoskeletal association of Sup35p and a possible second role for Sup35p (i.e. other than that as a translation termination factor). The C-terminal domain of Sup35p is highly homologous to elongation factor 1α (EF-1α), which is known to have an association with the cytoskeleton and interacts with actin (Kushnirov *et al.,* 1988; Murray *et al.,* 1996; Liu *et al.,* 1995). Mutations in the *SUP35* gene of *S.cerevisiae* can confer sensitivity to the anti-microtubule drug benomyl (Tikhomirova & Inge-Vechtomov, 1996) and have been reported to inhibit cell division (Kikuchi *et al.,* 1988). A cytoskeletal association of Sup35p raises the possibility that the prion conformer could form amyloid-like fibrils *in vivo,* which are integrated into the cell cytoskeleton. A cytoskeletal association of Sup35p might ensure the segregation of the *[PSI⁺]* determinant, both in its normal pattern of inheritance and in its elimination from a strain, upon growth in the presence of GuHCl.

3.5 Materials and Methods

3.5.1 Strains

The genotype of the strain used in this study was:

BSC783/4a: *[PSI⁺], SUQ5, ade2-1, ura3-1, his3-11, his3-15, leu2-3, leu2-112, MATa*

3.5.2 Growth media

BSC783/4a was grown at 30°C, on ¼YEPD solid medium (4% w/v glucose, 1% w/v Bacto-peptone, 0.25% w/v yeast extract, 2% w/v agar). Most liquid cultures were also grown at 30°C, in YEPD complete medium (2% w/v glucose, 1% w/v Bacto-peptone, 1% w/v yeast extract), with or without 3mM GuHCl. For studies using ethanol-supplemented media, strains were grown in flasks, sealed with rubber bungs, to reduce evaporation of the alcohol from the medium.

3.5.3 Thermotolerance assays

In preliminary experiments (Figure 3a), cultures were grown at 25°C to mid-exponential phase in YEPD (OD_{600} 0.4) and then adjusted to a density of approximately 3.5 x 10^3 cells per mL, by dilution in the appropriate YEPD media. Cultures were then exposed to a one hour pretreatment; the addition of GuHCl to 3mM, EtOH to 3%v/v, or a shift to 37°C. Strains were then transferred to a 52°C shaking water bath and aliquots were removed and stored on ice. 150μL aliquots (approximately 500 cells) were plated in triplicate, onto solid YEPD medium, (2% w/v glucose, 1% w/v Bacto-peptone, 1% w/v yeast extract, 2% w/v agar) and viable counts were determined after 3 days growth at 30°C. Note that the viable counts were not performed on ¼YEPD solid medium. For the thermotolerance assays, incorporating a washing procedure (Figure 3b), cultures

were adjusted to a density of approximately 3.5 x 10^5 cells per mL, prior to any pre-treatment. Following a 1hr pre-treatment, culture aliquots were diluted 100-fold using sterile water and incubated at 25°C for 10 minutes. The assays were then performed as described previously.

3.5.4 Sedimentation analysis of Sup35p

Exponential cultures were grown to an OD_{600} of 0.5, whereupon 15ml culture aliquots were harvested. 15mL aliquots of stationary phase cultures were also harvested, following their dilution to an OD_{600} of 0.5. cell pellets were lysed by glass beads at 4°C, in 500µL buffer (25mM Tris-HCl pH 8, 150mM NaCl, 5mM EDTA, 5mM PMSF, Complete protease inhibitor mix [Boehringer Mannheim, 1 tablet per 5ml buffer]). Whole-cell lysates were extracted from the glass beads, in the complete absence of centrifugation. Lysates were then clarified, by centrifugation at 4°C for 10 minutes at 13000 x g. Following the addition of 5 x reducing SDS-PAGE sample buffer (2.5mL glycerol, 1.25mL 20% v/v SDS, 0.25mL 2-mercaptoethanol, 1.25mL 0.5M Tris-HCl pH 6.8), samples of whole-cell lysates and clarified lysates were boiled for 10 minutes, cooled on ice and then spun for 5 minutes at 13000 x g. Protein samples were separated using 10% SDS-polyacrylamide gels and electrophoretically transferred to nitrocellulose. Protein blots were probed using polyclonal sera raised against yeast Sup35p and Sup45p expressed in *E.coli*, as previously described (Stansfield *et al.*, 1995a) and against yeast Hsp104p (Stressgen, UK).

3.5.5 Mathematical expression of a segregational model

To test the segregational hypothesis, Ruddock (in preparation) devised a mathematical expression for curing within the framework of the model. The segregational model was founded upon the assumptions that; (1) GuHCl totally inhibits prion-seed replication, (2) seeds are randomly segregated between mother and daughter cells, with each computational step in the model leading to a Poisson distribution of seeds, (3) seeds are

stable (i.e. the total number of seeds is constant within a GuHCl-containing culture) and (4) a single seed elicits a *[PSI⁺]* phenotype. The line of best fit to a simple segregation model for curing was calculated as follows. A simple iterative computer model was generated where an initial population of cells, each with n *[PSI⁺]* seeds, was followed through successive generations. For each generation the percentage of cells with n, n-1, n-2, ...2,1,0 prion seeds was calculated, assuming a simple random distribution of seeds, between parent and daughter cells (i.e. the average number of *[PSI⁺]* seeds per cell decreases two-fold with each generation). Since the phenotype is assumed lost only when a cell is completely free of prion seeds, a plot of generation vs. percentage of cells with 0 prion seeds (i.e. % *[psi⁻]*) gives a plot of curing for any initial value of n. However, such a model only generates a plot for integral numbers of generations. It is possible to generate an estimate for fractional generations by looking at variable values for n (where n>16). For example, the plot for n=16 is to a first approximation the same as the plot for n=32, but shifted by one generation. Hence, the plot for n=28 is effectively the plot for n=32, shifted by 0.19 generations. Thus, a curve may be generated for n=32 (by using integral values of n=16 to 32) which has both integral and fractional values for generation number.

A line of best fit may be generated for each of the curing data sets, by comparing the values of the experimentally determined % *[psi⁻]* with those values derived from the model. A plot of the corresponding generation number (from the model) and time (from the experiment) gives a straight-line plot (Figure 3.7). The gradient of this plot yields a measure of the generation time and the intercept gives a measure of the shift in generations required, from the experimental value for n to the model value of n=32. For each set of experimental data, only values in the range between 2-98% *[psi⁻]* were used for the best fit. The line of best fit for each set of data gave a calculated generation time that was within 10% of that determined experimentally.

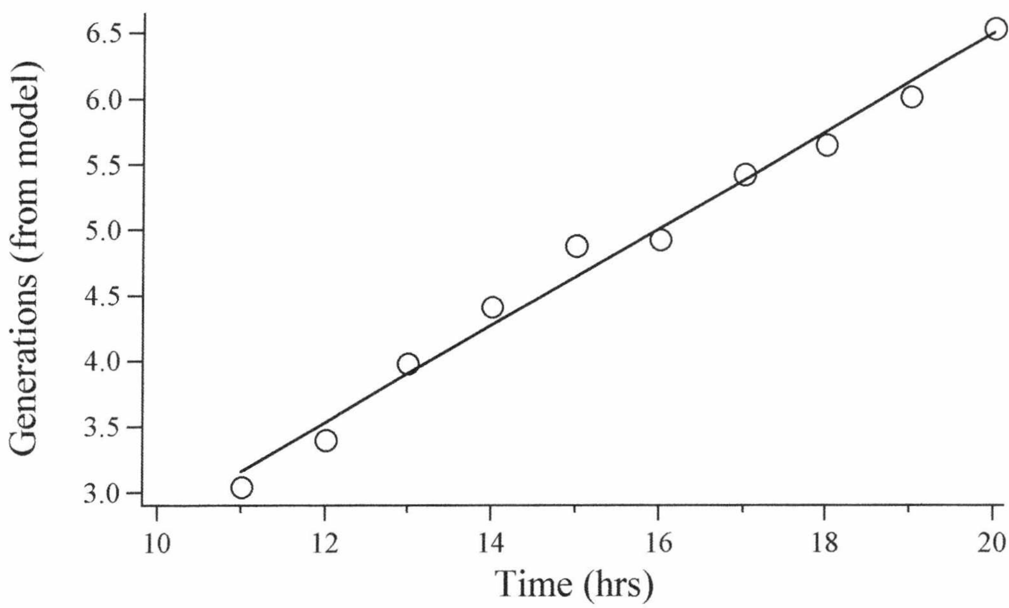


Figure 3.7 Linear plot of the number of generations against growth time, for one set of curing data in the presence of 3mM GuHCl. The number of generations was obtained by comparing those values of % *[PSI⁺]* determined experimentally at each time point, with those calculated from an iterative model for curing by random segregation, in which the initial number of prions (n) was set at 32. Only values of % *[PSI⁺]* between 98 and 2 were considered. The line of best fit gives values for the gradient of 0.373 and for the intercept of -0.934, with R^2=0.982. The gradient represents 1/generation time, yielding a value for calculated generation time of 2.68 hrs. This compares well with the experimentally determined generation time of 2.5 hrs. The intercept gives a measure of the generation shift from the real number of prion seeds to that used in the iterative model. The best-fit number of prion seeds can be calculated by 32 x ($2^{-\text{generation shift}}$), yielding a value of 61. Similar linear plots for other curing curves in each case yielded generation times within 10% of the experimentally determined doubling time and an average number of prion seeds of 62 $\pm$ 10.

Chapter IV

A model for the mechanism of elimination of the yeast prion-like determinant *[PSI⁺]* by guanidine hydrochloride

Chapter

A hypothesis for the mechanism of [PSI⁺] curing by guanidine hydrochloride

4.1 Abstract

The *[PSI⁺]* allosuppressor phenotype of the yeast *Saccharomyces cerevisiae* is a manifestation of the prion-like properties of the chromosome-encoded protein Sup35p. *[PSI⁺]* strains exhibit a marked decrease in the efficiency of translation termination, but do not exhibit any gross physiological defect. Strains can be 'cured' of *[PSI⁺]* by growth in the presence of millimolar concentrations of guanidine hydrochloride (GuHCl). Previously, we have demonstrated that GuHCl inhibits a critical event in the propagation of the prion-conformer of Sup35p (see Chapter 3). We proposed that GuHCl might elicit the elimination of the *[PSI⁺]* determinant by influencing the behaviour of either Sup35p itself or Hsp104p, thereby precluding prion replication. Plausibly, GuHCl might inhibit the 'refolding' activity of Hsp104p (Glover & Lindquist, 1998) or it may stabilise native Sup35p, or its prion conformer. Here an alternative hypothesis is proposed, namely that the guanidinium cation inhibits the activity of a third, as yet unidentified, protein that mediates the post-translational modification of an arginine residue(s) within nascent Sup35p. The guanido moiety is present at the terminus of arginine side chains and in rare examples, can be the site of a post-translational modification of proteins *in vivo*. As well as GuHCl, several other guanidinium salts were shown to possess a potent curing ability. A variety of guanido-containing compounds were tested for their ability to cure *[PSI⁺]* but none were able to inhibit prion replication. Nonetheless, using isoelectric focussing, it was shown that the addition of GuHCl to growth media does alter the biophysical properties of Sup35p, which is consistent with a change in its post-translational modification. We discuss the possible significance of an arginine modification of Sup35p in relation to *[PSI⁺]* and suggest how the proposed 'arginine model' might be further tested.

4.2 Introduction

The prion protein (PrP) is thought to be the sole component of the infectious scrapie agent and the principal factor in the family of neurodegenerative diseases, termed the spongiform encephalopathies (reviewed in Cohen & Prusiner, 1998; Prusiner *et al.*, 1998). The unusual genetic properties of two non-Mendelian elements, namely *[PSI⁺]* and *[URE3]*, lead to the proposal that prions serve as a vehicle of phenotypic information in the yeast *Saccharomyces cerevisiae* (Cox, 1994; Wickner, 1994). Biochemical evidence has supported the hypothesis that the *[PSI⁺]* phenotype reflects the prion-like properties of the *SUP35* gene product, namely Sup35p (Patino *et al.*, 1996; Paushkin *et al.*, 1996; Glover *et al.*, 1997; King *et al.*, 1997; Paushkin *et al.*, 1997a). In a *[PSI⁺]* background, the normal cellular Sup35p is corrupted by the prion form and adopts the same non-functional prion conformation. Native Sup35p is known to be one of two eukaryote polypeptide release factors that mediate translation termination (Stansfield *et al.*, 1995a, Zhouravleva *et al.*, 1995). In a *[PSI⁺]* background, most Sup35p exists as large aggregates (Patino *et al.*, 1996; Paushkin *et al.*, 1996). This results in the intracellular depletion of functional termination factors (Paushkin *et al.*, 1996, 1997b; reviewed in Tuite & Lindquist, 1996), which manifests as an allosuppressor phenotype (i.e. inefficient translation termination in the presence of a nonsense suppressor tRNA).

Tuite *et al.* (1981a) demonstrated that *[PSI⁺]* could be eliminated, or 'cured', from yeast by growth in the presence of the protein denaturant guanidine hydrochloride (GuHCl). Remarkably, *[PSI⁺]* strains are cured, with near total efficiency, by 1-5mM GuHCl (Tuite *et al.* 1981a). Moreover, GuHCl has also been shown to cure yeast of two other prion-like determinants, namely *[URE3]* and *[PIN⁺]* (Wickner, 1994; Derkatch *et al.*, 1997). We have previously investigated the curing of *[PSI⁺]* by GuHCl (see Chapter 3) and demonstrated that GuHCl elicits the complete inhibition of prion replication. A most attractive hypothesis is that GuHCl elicits prion-loss by ablating the activity of Hsp104p, a stress protein essential for *[PSI⁺]*-maintenance (Chernoff *et al.*, 1995a). Hsp104p assembles into a homohexamer and facilitates the 'disaggregation' of polymeric stress-denatured protein (Parsell *et al.*, 1994a, b; Glover & Lindquist, 1998). A deletion of the *HSP104* gene or the expression of a non-functional mutant protein eliminates the *[PSI⁺]*

phenotype (Chernoff *et al.*, 1995a). *HSP104* is a so-called P*si No More (PNM)* gene (McCready *et al.*, 1977), with the wild type gene product being required for *[PSI⁺]* maintenance. The mutant protein bears two amino acid substitutions, which 'destroy' the two nucleotide-binding (NBD) sites of Hsp104p (Parsell *et al.*, 1991). A single mutation (K620T) in the C-terminal NBD eliminates oligomerisation of Hsp104p, but has little effect upon the hydrolysis of adenine nucleotides (i.e. the ATPase activity of Hsp104p). Whilst a single mutation in the first NBD (K218T) completely ablates the ATPase activity of Hsp104p, this mutant protein can still assemble into a homohexamer (Parsell *et al.*, 1994b). Either of the single mutations eliminates the ability of Hsp104p to confer stress tolerance to yeast (Parsell *et al.*, 1991) or to promote the resolubilisation of protein aggregates (Parsell *et al.*, 1994a). Glover & Lindquist (1998) have demonstrated that Hsp104p is extremely sensitive to GuHCl, supporting the hypothesis that GuHCl cures *[PSI⁺]* by inactivating Hsp104p (i.e. equivalent to a disruption of the *HSP104* gene).

Like *[PSI⁺]*, the extrachromosomal determinant *[PIN⁺]* is also cured by GuHCl and by the deletion of the *HSP104* gene (Derkatch *et al.*, 1997). Whilst this is consistent with the hypothesis that GuHCl cures yeast prion determinants by inactivating Hsp104p, it is reported that *[PIN⁺]*, unlike *[PSI⁺]*, is insensitive to overexpression of Hsp104p (Derkatch *et al.*, 1997). Furthermore, whilst GuHCl is known to cure the yeast determinant *[URE3]* (Wickner, 1994), the deletion or overexpression of Hsp104p has no effect upon *[URE3]* maintenance (C. Cullin, *pers. comm.*). These data have led us to a novel hypothesis that accounts for the ability of GuHCl to eliminate yeast prion determinants, but does not encompass Hsp104p as the 'target' for inactivation. We postulate that GuHCl acts as a competitive inhibitor of an arginine-modifying enzyme. We propose that at millimolar concentrations, GuHCl blocks the action of this putative *PNM* protein. In the absence of a critical arginine modification, we suggest that native Sup35p is unable to adopt the prion conformation, thereby resulting in the inhibition of replication of the *[PSI⁺]* determinant and the subsequent loss of the *[PSI⁺]* phenotype (Figure 4.1).

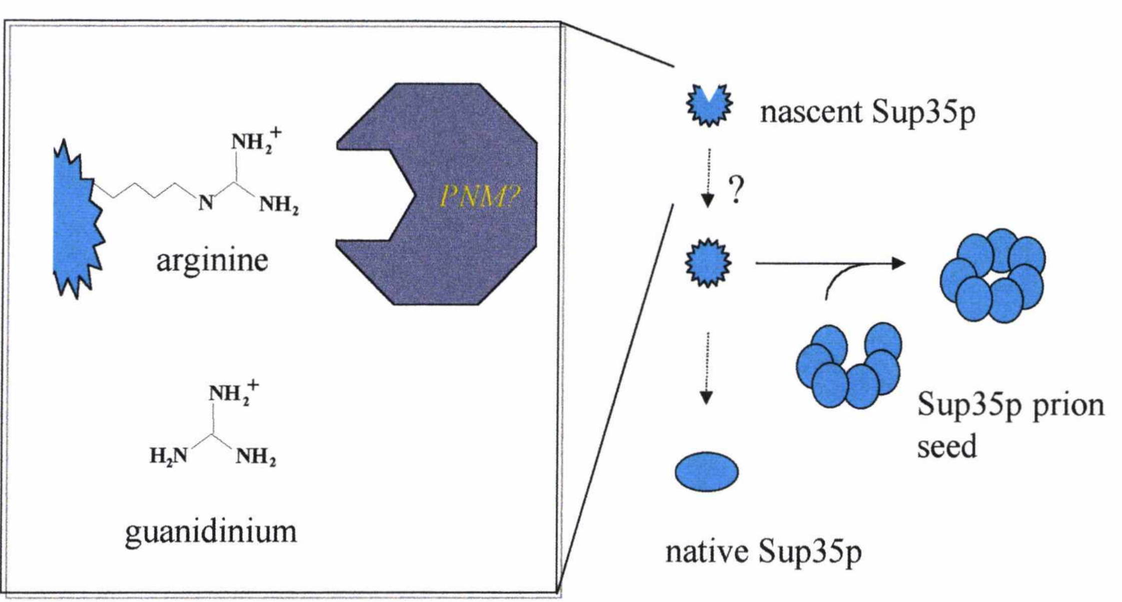


Figure 4.1 A model for the curing of yeast prions by GuHCl. It is proposed that the propagation of the prion conformer of Sup35p requires a post-translational modification of an arginine residue. This modification is catalysed by a putative enzyme, (*PNM?*), whose activity is inhibited by the guanidinium cation (Gdm^+). The modification of nascent Sup35p permits an intermediate conformation that can be 'sequestered' by a prion seed. However, in the presence of Gdm^+ (a competitive inhibitor of *PNM?*) an arginine within nascent Sup35p is not modified and therefore unable to adopt the aberrant conformation. Thus, prion replication is precluded and the culture is cured in a growth-dependent manner (see Chapter 3).

At physiological pH, the basic δ-guanido group of arginine is protonated. With three non-polar methylene groups and a charged, planar, guanido terminus, arginine has a 'bulky' side chain. As such, it seems plausible that an arginine side chain could disrupt protein-protein interactions. Whilst the prion-like properties of Sup35p and Ure2p (i.e. the *[PSI⁺]* and *[URE3]* protein determinants) do not share a common requirement of Hsp104p, both possess arginine residues within their prion forming domains [PFD's] (Kushnirov *et al.*, 1988; Coschigano & Magasanik, 1991; Ter-Avanesyan *et al.*, 1994; Masison *et al.*, 1997; see section 1.4.1). The post-translational modification of an arginine residue within the PFD of Sup35p (and indeed Ure2p) might result in the reduction of its 'bulkiness' and/or the removal of the charged guanido group. Such a modification may be required to eliminate a steric and/or an electrostatic barrier that precludes the self-association of Sup35p and ultimately, the replication of the *[PSI⁺]* determinant.

Two preliminary experiments were therefore performed to test the 'arginine model'. First, it was investigated whether other compounds that contain a guanido moiety are able to cure *[PSI⁺]*. Second, the biophysical properties of Sup35p produced by strains growing in liquid medium alone, or in medium containing GuHCl were compared. Patino *et al.* (1996) have reported that Sup35p exhibits the same biophysical properties (as determined by two-dimensional gel electrophoresis) when produced in either a *[PSI⁺]* or a *[psi⁻]* background. Within the framework of the arginine model, we predict that the biophysical properties of Sup35p will be different when the protein is produced in the presence of GuHCl. Regardless of *[PSI⁺]*-status, the same modified species of Sup35p would be produced in cells growing in normal medium. However, the addition of GuHCl is predicted to elicit the production of a second non-modified species of Sup35p. One-dimensional isoelectric focussing was employed to test these predictions.

4.3 Results

4.3.1 Guanidine derivatives do not cure *[PSI⁺]*

A variety of compounds were tested for their ability to eliminate the *[PSI⁺]* determinant from the yeast strain BSC 783/4a. The compounds were selected on the criteria that they contained the guanido functional group or they were known to be modifiers of protein structure (Figure 4.2). BSC 783/4a *[PSI⁺]* was grown at 30°C for 5 days on solid ¼YEPD medium, supplemented with a test compound. The curing potential of these compounds was assessed on the basis that the *[PSI⁺]* strain grows to form white colonies on solid rich medium, but gives rise to red colonies when devoid of the prion determinant [i.e. cured *[psi⁻]* strains are red] (Cox, 1965; Table 4.1).

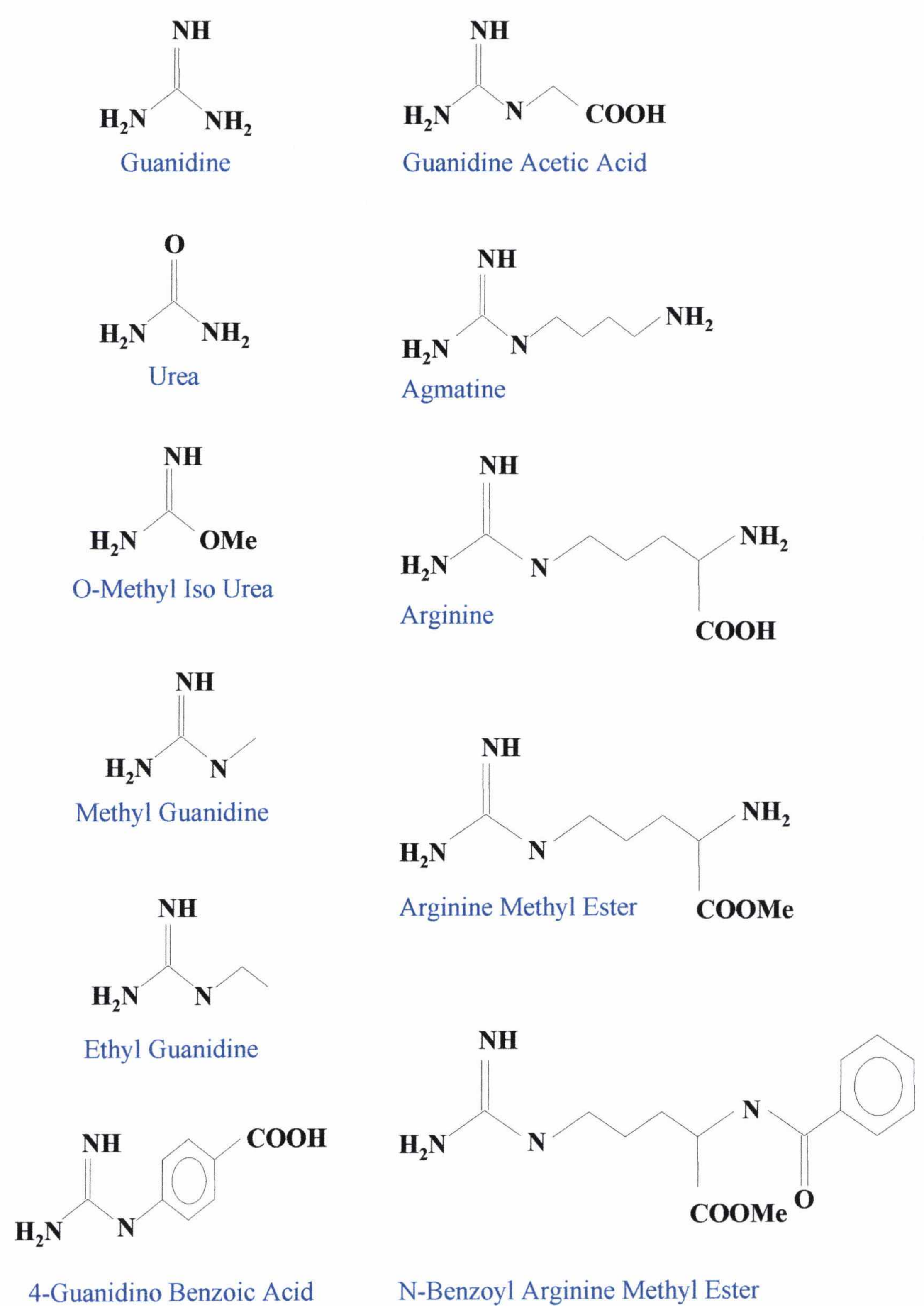


Figure 4.2 Reagent screen for *[PSI⁺]* curing compounds. Various guanidinium salts, guanido-containing molecules and protein denaturants were selected for use in a *[PSI⁺]* curing assay.

The results of the compound screen were unequivocal. Of all the reagents tested, only the guanidine salts cured $[PSI^+]$ and did so with near-total efficiency (Table 4.1). Except for the guanidine salts, the frequency of $[PSI^+]$ to $[psi^-]$ conversion in the presence of the test compounds was less than 0.5% for a total of ~500 colonies per plate (data not shown).

Table 4.1 Screen for reagents that eliminate $[PSI^+]$. Strain BSC 783/4a $[PSI^+]$ was grown on solid ¼YEPD medium, supplemented with the test compound. Curing was seen as the growth of red $[psi^-]$ colonies, rather than white $[PSI^+]$ colonies (Cox, 1965).

Compound		Concentration (mM)	Elimination of $[PSI^+]$
Guanidine hydrochloride	Gu·HCl	5	✓
Guanidine hydrogen thiocyanate	Gu·HSCN	5	✓
Guanidine dihydrogen sulfate	$(Gu)_2 \cdot H_2SO_4$	5	Lethal
		2.5	✓
Methyl guanidine hydrochloride	MeGu·HCl	5	Lethal
		2.5	✗
Ethyl guanidine dihydrogen sulfate	$(EtGu)_2 \cdot (H_2SO_4)_2$	5	Lethal
		2.5	✗
Guanidine acetic acid	GuAcOH	5	✗
4-Guanidinobenzoic acid hydrochloride	GuBzOH·HCl	5	✗
Agmatine dihydrogen sulfate	$Gu(CH_2)_4NH_2 \cdot H_2SO_4$	5	✗
		25	✗
Arginine	Arg	5	✗
Arginine methyl ester	Arg(OMe)	5	✗
N-benzoyl arginine methyl ester	(BzO)Arg(OMe)	5	✗
Urea	$(NH_2)_2CO$	10	✗
O-Methyl iso Urea	$NH_2C(OMe)NH$	5	✗

As is the case with the δ-guanido group of arginine, guanidine is extremely basic and predominantly exists as the guanidinium (Gdm^+) cation in liquid medium. The addition of a guanidine salt, to yield a final concentration of 5mM Gdm^+, elicits a slight inhibition of growth of strain BSC 783/4a (data not shown). However, when added to a final concentration of 10mM Gdm^+, the guanidine salts were lethal to the strain (Table 4.1). Plausibly, the accumulation of Gdm^+ might permit an intracellular concentration (i.e. $\geq$ 5mM) that alters the structure of a protein, such that prion replication is precluded. However, the narrow concentration window between a slight inhibition of growth and lethality (i.e. 5-8mM Gdm^+; data not shown) suggests that this is not the case and that the intracellular concentration of Gdm^+ is the same, or possibly lower than that of the culture medium.

None of the guanidine 'derivatives' tested demonstrated an ability to cure *[PSI⁺]* (Table 4.1). The simple methyl derivative of guanidine was extremely toxic, when present at a final concentration of 5mM, but did not cure when used at lower concentrations. Various derivatives of arginine were employed in the screen, to address the steric and electrostatic criteria for a curing compound. However, none of these compounds cured *[PSI⁺]*, even when employed at higher concentrations (e.g. agmatine at 25mM, Table 4.1). The failure of the arginine derivatives to cure *[PSI⁺]* might be construed as evidence that the arginine model is incorrect. However, it is plausible that these compounds are simply modified by *PNM?* and therefore no longer able to behave as competitive inhibitors. Within the framework of the arginine model, these data suggest that Gdm^+ is able to cure *[PSI⁺]* since it has an extremely high affinity for the putative modifying enzyme but is not itself a substrate for modification.

Alternatively, the guanido-containing compounds might be metabolised by yeast and thus exhibit poor curing properties. *In vivo,* arginine is a key component of the urea cycle and an important factor in several metabolic processes (Cohen, 1981; reviewed in Cooper, 1982). The enzyme arginase catalyses the catabolism of arginine, to yield ornithine and urea (reviewed in Whitney & Magasanik, 1973 and Cooper, 1982). The reaction effectively converts the guanido group of arginine to a primary amine (Figure 4.3). Importantly, the reaction does not generate free Gdm^+ cations. Thus, it is plausible that the arginine-like compounds employed in the screen (Figure 4.2), failed to cure *[PSI⁺]*

because they were metabolised by arginase, or simply because they were 'processed' by the *PNM?* protein itself.

H_2N NH N NH_2 COOH $\xrightarrow[\text{Arginase}]{H_2O}$ H_2N NH_2 COOH + NH_2 O NH_2

Arginine Ornithine + Urea

Figure 4.3 Arginase catalyses the conversion of arginine to ornithine. Arginase may eliminate the curing properties of guanido-containing compounds (Figure 4.2) by catalysing the conversion of this functional group to a primary amine.

4.3.2 Overexpression of Sup35p in yeast

In an alternative approach to testing the arginine-modifying model, Sup35p was analysed when produced by yeast grown in normal media alone or in media supplemented with GuHCl. To facilitate such analysis, the plasmid pUKC1501 was constructed (see section 4.5.3) that carries the *SUP35* gene, under the control of the galactose-inducible *GAL1* promoter (St John & Davis, 1979, 1981). The *[psi⁻]* variant of strain BSC 783/4a was normally employed in the overexpression experiments to avoid a 'slow-growth' phenotype that is associated with the overexpression of Sup35p in a *[PSI⁺]* strain (Dagkesamanskaya & Ter-Avanesyan, 1991). The inhibition of growth, exhibited by these cells is thought to result from the severe depletion of soluble translation termination factors (Paushkin *et al.*, 1997b; Derkatch *et al.*, 1998).

Typically, transformants of strain BSC 783/4a were grown as starter cultures in minimal media (i.e. with glucose as the carbon source). The expression of genes downstream of the *GAL1* promoter is tightly repressed in the presence of glucose (St John & Davis,

1981). The 100-fold dilution of a starter culture, into glucose-free induction media and its growth for 24hrs at 30°C achieved Sup35p overexpression (Figure 4.4).

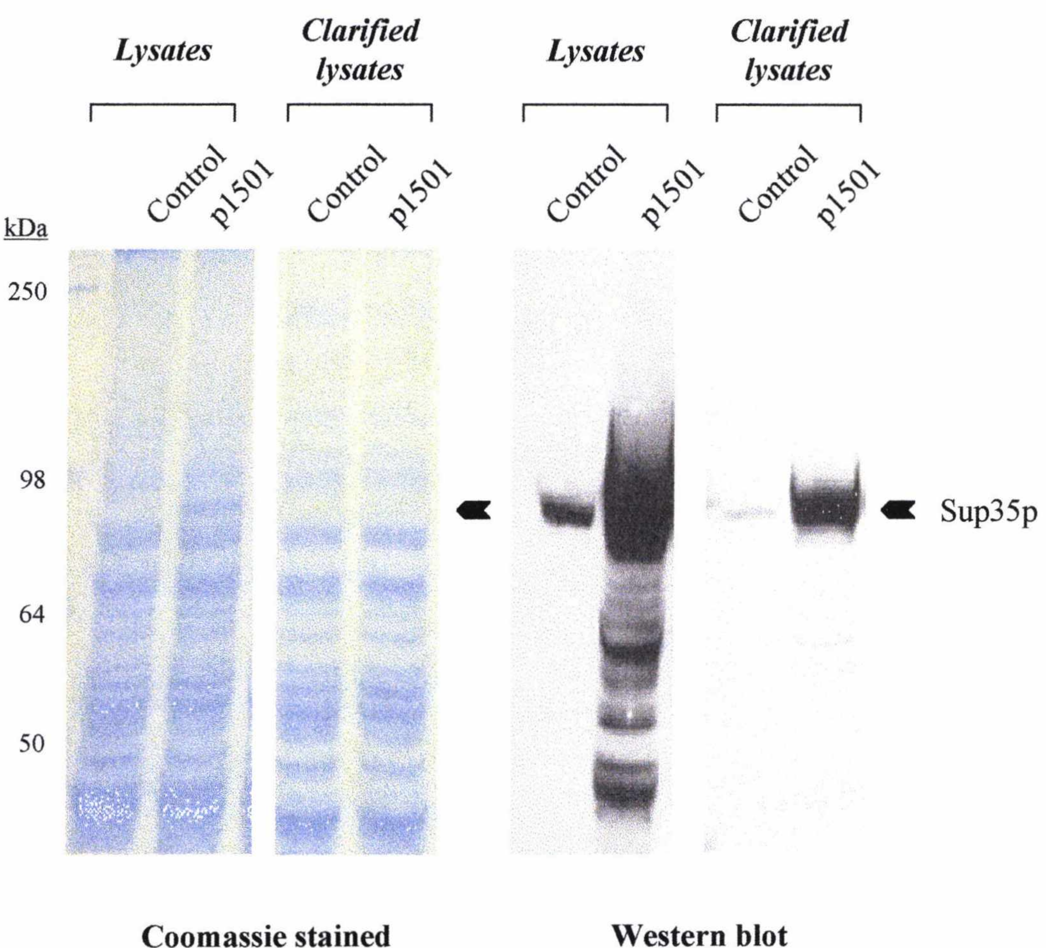


Figure 4.4 SDS-PAGE and Western blot analysis of Sup35p overexpression in yeast. Strain BSC 783/4a *[psi$^-$]*, bearing either the control vector pUKC639 (K.M. Jones, unpublished) or pUKC1501 (*SUP35* cloned into pUKC639, downstream of the *GAL1* promoter; this study) was grown for 24hrs at 30°C in galactose induction medium. Culture aliquots were harvested, lysed and clarified by centrifugation at 13 000 x g for 10min. Samples of whole cell lysates and clarified lysates were then analysed by SDS-PAGE. Gels were either stained with coomassie blue or transferred to nitrocellulose. Western blot analysis was performed using a rabbit polyclonal antiserum, raised against recombinant Sup35p produced in *Escherichia coli.*

4.3.3 Biochemical characterisation of Sup35p species

It was noted that whilst the overexpression permitted the detection of Sup35p in whole cell lysates, by SDS-PAGE and coomassie blue staining, a large fraction of the protein sedimented with the cell-debris that was removed by centrifugation (Figure 4.4). Whilst the level of expression was greatly increased, with respect to endogenous levels, this loss of Sup35p was a severe hindrance to the purification of soluble Sup35p by standard liquid chromatography techniques. The majority of the overexpressed Sup35p was associated with the 'particulate' fraction of a cell lysate, which cannot be applied to standard chromatography systems. Sup35p could be isolated from clarified lysates (i.e. soluble fraction) using cation exchange chromatography, when performed at pH 6 and at a concentration of sodium chloride below 250mM (as detected by Western blot; data not shown). However, the level of soluble Sup35p isolated from cells grown in 3 litres of culture (final OD_{600} ~2.0) was too low, even in cation exchange fractions, to be detected by coomassie staining (data not shown). Since Sup35p was not a significant component of these concentrated fractions, this approach was not pursued as a source of Sup35p for further experimentation.

Although the biophysical analysis of pure Sup35p is ultimately required to test the arginine-modification hypothesis, one-dimensional isoelectric focussing (1D IEF) was used to compare the biophysical properties of Sup35p, produced by yeast growing in normal liquid medium or in medium containing 3mM GuHCl (Figure 4.6). The quantity and 'solubility' of Sup35p, produced by variants of strain BSC 783/4a, was first analysed by SDS-PAGE and Western blot (Figure 4.5). It is noteworthy that the *[PSI⁺]* determinant does not preclude the overexpression of Sup35p but that it eliminates the increased production of 'soluble' Sup35p.

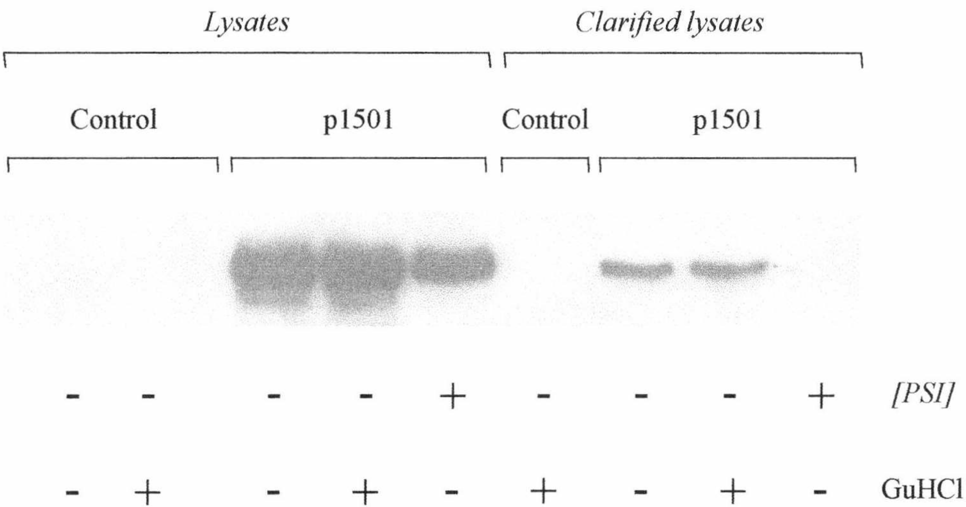
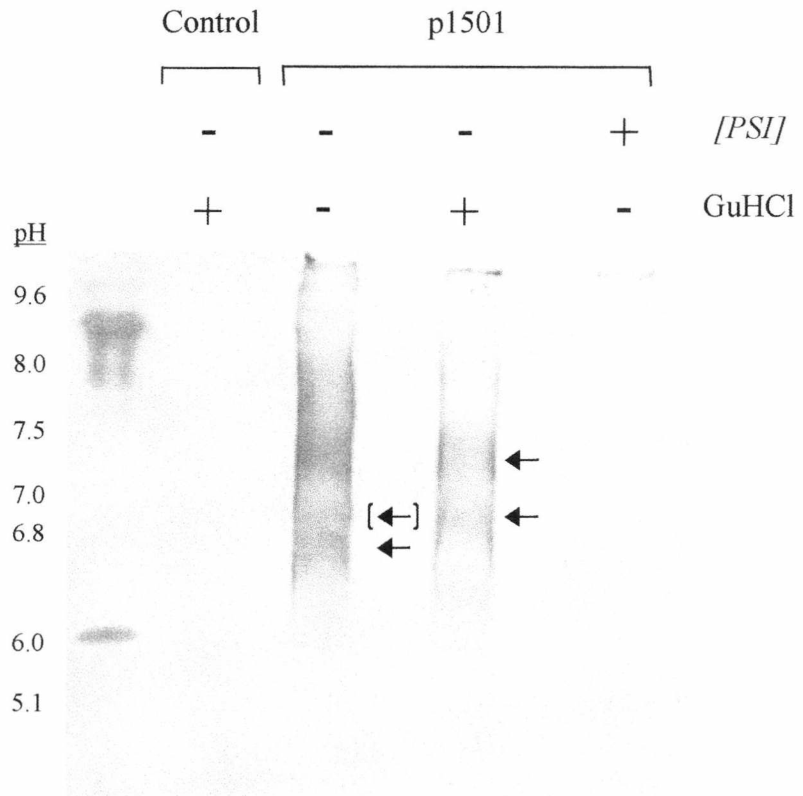


Figure 4.5 Influence of GuHCl and *[PSI⁺]* status upon the expression and solubility of Sup35 overexpressed in yeast. Variants of strain BSC 783/4a were grown at 30°C for 24hrs in galactose induction medium alone, or supplemented with 3mM GuHCl. Strains were transformed with either the control vector pUKC639 (K.M. Jones, unpublished) or with pUKC1501 (*SUP35* cloned into pUKC639, downstream of the *GAL1* promoter; this study). Culture aliquots were harvested, lysed and clarified by centrifugation at 13 000 x g for 10min. Samples of whole cell lysates and clarified lysates were then analysed by SDS-PAGE and Western blot, using a rabbit polyclonal antiserum raised against recombinant Sup35p. Note that whilst endogenous Sup35p is not visible in the control lanes, it was detected after prolonged exposure of the detector film (data not shown).

Two predominant species of Sup35p were produced, under normal growth conditions that exhibited isoelectric points (pI's) of approximately 7.3 and 6.6 (Figure 4.6). Although of relatively poor resolution, there also appeared to be a third species, with a pI of approximately 6.9. Intriguingly the theoretical pI of Sup35p is 6.8, which suggests that this minor species is actually nascent Sup35p and that the two major species (pI ~7.3 and ~6.6) might be post-translationally modified species.



<u>Figure 4.6</u> GuHCl alters the biochemical properties of Sup35p in yeast. The variants of strain BSC 783/4a (transformed with the vector pUKC639; K.M. Jones, unpublished) and the Sup35p-overexpressing strain (BSC 783/4a *[psi$^-$]* transformed with pUKC1501) were grown at 30°C for 24hrs in induction medium, in the presence or absence of 3mM GuHCl. Soluble extracts of cells were applied to a one-dimensional isoelectric focussing gel, following the centrifugation of whole cell lysates at 13 000 x g for 10min. Soluble proteins were then focussed within a non-linear pH gradient and transferred to nitrocellulose for Western blot analysis, using a Sup35p-specific rabbit polyclonal serum.

Sup35p was also detected as two major species when produced in the presence of GuHCl, although the pI's of these species was ~7.3 and ~6.9 (Figure 4.6). Thus, GuHCl brought about a change in the biophysical properties of Sup35p *in vivo*, as predicted by the arginine modification model. GuHCl may therefore promote the accumulation of the nascent Sup35p (pI ~6.9) by preventing the formation of a covalently modified species (of pI ~6.6). However GuHCl did not influence the production of a second modified Sup35p species, whose pI was ~7.3.

4.4 Discussion

A novel hypothesis is proposed to describe the mechanism by which the protein denaturant GuHCl eliminates the yeast prion determinant *[PSI$^+$]*. The caveat of the arginine model is that the guanidinium cation is an efficient competitive inhibitor of an enzyme, termed *PNM?*, whose activity is essential for the maintenance of *[PSI$^+$]*. We propose that *PNM?* is required for the modification of a *Psi No More* protein (i.e. a protein necessary for *[PSI$^+$]* maintenance) and that *PNM?* catalyses the post-translational modification of arginine residues within nascent polypeptides. In the simplest scenario, the substrate for *PNM?* is Sup35p itself (Figure 4.1) and the modification of an arginine residue (or residues) is a prerequisite for the self-association of Sup35p prion conformers.

Arginine modifications in proteins are rare, but are found to occur in both prokaryotes and eukaryotes. These post-translational alterations are confined to specific proteins and apparently for specific reasons (reviewed in Springer *et al.*, 1979; Wold, 1981; Aleta *et al.*, 1998). The most common modification of arginine in eukaryotes is that of ADP-ribosylation and this can serve a regulatory role, a fact fully exploited by enterotoxigenic bacteria that produce ADP-ribosylating toxins (reviewed in Spangler, 1992; Burnette, 1994). Methylation of arginine residues is a feature of cellular stress responses (Desrosiers & Tanguay, 1988), ageing of proteins (Najbauer *et al.*, 1996) and signal transduction in eukaryotes and prokaryotes (reviewed in Springer *et al.*, 1979; Aleta *et al.*, 1998).

Two preliminary experiments have been performed to test the arginine model, whereby the activity of an arginine-modifying enzyme is required for the propagation of a yeast prion protein. Firstly, a variety of compounds were screened for their ability to cure *[PSI$^+$]*. Whilst many of those compounds contained the guanido functional group (see Figure 4.2 above), only guanidine salts exhibited a curing ability (see Table 4.1 above). These data have been interpreted as evidence that these compounds are metabolised by yeast and that they might serve as substrates, rather than inhibitors, of *PNM?*. Alternatively, it is known that *in vivo,* the enzyme arginase catalyses the hydrolysis of arginine (and presumably arginine derivatives) to yield urea and ornithine (Whitney &

Magasanik, 1973). This catabolic reaction eliminates the guanido functional group of arginine and would thus destroy any potential inhibitory (curing) activity of the compound. To address this issue, the compounds could be re-screened in a curing assay in which the *[PSI⁺]* strain was grown on ammonia or glutamine as the sole nitrogen source. Such conditions elicit an extremely low level of arginase production (Magasanik, 1992) and would thus be expected to enhance any curing activity of the arginine derivatives.

The arginine model also predicts that GuHCl will elicit a change in the biochemical properties (i.e. covalent modification) of a *Psi Mo More* protein. One-dimensional isoelectric focussing has been employed to test whether GuHCl alters the composition of Sup35p. The data suggest that Sup35p exists as two distinct covalently-modified species under normal growth conditions and that GuHCl does preclude the production of one of these species (see Figure 4.6 above). In the presence or absence of GuHCl, nascent Sup35p (pI ~6.9) is modified to yield a species of pI ~7.3 (Figure 4.6). However, GuHCl appeared to promote the accumulation of nascent Sup35p, with a concomitant decrease in the abundance of a second modified species, pI ~6.6.

Whilst a protocol was established for the overexpression of Sup35p in yeast, the isolation of the protein in a form suitable for extensive biophysical analysis was not achieved. If such a protocol had been established, it should have been possible to identify the post-translational modifications of Sup35p using a combination of techniques that included reversed phase chromatography, mass spectrometry, proteolytic digestion and N-terminal sequencing. This target might be best achieved using two-dimensional (2D) electrophoresis. The recent requirement to link genomic and proteomic data has driven the development of both 2D gel equipment and strategies, to permit the purification and biophysical characterisation of a single protein species from complex mixtures of cellular proteins (Shevchenko *et al.*, 1996; Wilm *et al.*, 1996; reviewed in Humphrey-Smith *et al.*, 1997). The biochemical analysis of Sup35p species could be achieved using 2D gels as follows; cells overexpressing Sup35p could be lysed in a non-ionic detergent solution and resolved in two-dimensions (i.e. on the basis of charge and size) using thicker commercial gels. These newly available gels would eliminate the requirement for

detection of Sup35p by Western blot, since more whole cell lystate and therefore more Sup35p can be resolved, thereby facilitating the biophysical analysis of Sup35p species.

Intriguingly, the post-translational modification of arginine residues within mammalian PrP has been reported (Hope *et al.*, 1988a; Turk *et al.*, 1988; Safar *et al.*, 1990; Figure 4.7). However, these putative modified arginines do not occur within the minimal region required for infectivity and appear to have been ignored.

Sup35p

```
         10        20        30        40
MSDSNQGNNQQNYQQYSQNGNQQQGNNRYQGYQAYNAQAQ
         50        60        70        80
PAGGYYQNYQGYSGYQQGGYQQYNPDAGYQQQYNPQGGYQ
         90        100       110       120
QYNPQGGYQQQFNPQGGRGNYKNFNYNNNLQGYQAGFQPQ
SQGMS
```

PrP

```
         10        20        30        40
FVAMWTDVGLCKKRPKPGGWNTGGSRYPGQGSPGPNRYPP
         50        60        70        80
QGGGTWGQPHGGGWGQPHGGGWGQPHGGGWGQPHGGGWGQ
         90
GGGTHNQWNK
```

Figure 4.7 The N-terminal repeat-containing domains of Sup35p and PrP. The primary sequence of Sup35p [residues 1-125] (Kushnirov *et al.*, 1988; Wilson & Culbertson, 1988) and hamster PrP27-30 [partial sequence] (Oesch *et al.*, 1985) is shown. Arginine residues (R) within each sequence are highlighted. The first two arginine residues (**R**) within PrP27-30 have been reported as post-translationally modified (Hope *et al.*, 1988a; Turk *et al.*, 1988; Safar *et al.*, 1990).

In the case of the yeast prions, arginines are found within the prion forming domains of both Sup35p and Ure2p (see section 1.4.1) and as such, arginine modifications might be of much greater significance. For example, the arginine model predicts that the kinetics of *in vitro* fibril formation of Sup35p (Glover *et al.*, 1997; DePace *et al.*, 1998), purified

from yeast rather than *Escherichia coli*, would be remarkably different. Post-translationally modified Sup35p, isolated from yeast grown in the absence of GuHCl, is predicted to self-assemble much more rapidly into amyloid fibres than the unmodified protein (i.e. that isolated from *E.coli* or from yeast grown in GuHCl-containing media).

To complement the 'biophysical testing' of the arginine model, arginine-deficient mutants of Sup35p could be generated by site-directed mutagenesis. The substitution of one or both of the arginine residues (R28 and R98) within the prion forming domain of Sup35p (Figure 4.7) should generate proteins able to support *[PSI⁺]*, but insensitive to curing by GuHCl. In the absence of one or both arginine residues, it is predicted that there will be no 'barrier' to Sup35p self-assembly and therefore no requirement for a post-translational modification of an arginine residue. Hence, the activity of the putative modifying enzyme *PNM?*, becomes inessential for *[PSI⁺]* maintenance and thus no longer provides a mechanism for curing by GuHCl.

The arginine model offers a plausible, alternative, explanation for the mechanism by which GuHCl can eliminate the yeast prion determinant *[PSI⁺]*. The inhibition of Hsp104p function, by millimolar concentrations of GuHCl (Glover & Lindquist, 1998), would also be predicted to result in the elimination of the *[PSI⁺]* determinant from yeast (see section 3.4). However, the role that Hsp104p plays in the maintenance of the two other yeast prion elements, namely *[URE3]* and *[PIN⁺]*, remains unclear (Wickner, 1994; Derkatch *et al.*, 1997; C. Cullin, *pers. comm.)*. The arginine model can be extended to encompass the GuHCl-sensitivity of *[PSI⁺]*, *[URE3]* and *[PIN⁺]*, if it is assumed that the action of the putative arginine-modifying enzyme *PNM?* is a prerequisite for the propagation of the prion conformer of each of the appropriate protein determinants.

4.5 Materials and methods

4.5.1 Strains

The genotype of the *Saccharomyces cerevisiae* strain used in this study was:

BSC783/4a: *[PSI⁺], SUQ5, ade2-1, ura3-1, his3-11, his3-15, leu2-3, leu2-112, MATa*
The strain was rendered prion-free (i.e. *[psi⁻]*) by growth on solid defined minimal medium, supplemented with 3mM GuHCl (Tuite *et al.*, 1981a).

The genotype of the *Escherichia coli* strain used in this study was:
JM109: F', *traD36, $lacI^q\Delta(lacZ)$, M15, proAB/recA1, endA1, gyr96, (Nal^r), thi, hsdR17, (r_k –m_k+), supE44, relA1, Δ(lac-proAB)*
This strain was employed throughout this work for plasmid manipulation.

4.5.2 Growth media

Yeast strains were grown at 30°C, either in liquid or on solid (+2% w/v agar) defined minimal medium: 2% w/v glucose, 0.67% w/v Difco defined minimal medium without amino acids, supplemented with the appropriate amino acids and cofactors: 20mg/L except adenine which was added at 200mg/L. For the galactose-induction experiments, all liquid media was adjusted to pH 7, by the addition of sodium hydroxide solution. Unadjusted medium had a low pH (i.e. <5) and did not promote the overexpression of the *SUP35* gene (data not shown). Induction of the *GAL1* promoter was achieved by a 1/100 dilution of a starter culture into galactose induction media (pH 7): 2% w/v galactose, 0.67% w/v Difco defined minimal medium without amino acids, supplemented with the appropriate amino acids and cofactors: 20mg/L except adenine which was added at 200mg/L.

The *E.coli* strain JM109 was grown at 37°C in Luria-Bertani (LB) medium (0.5% w/v yeast extract, 1% w/v tryptone, 0.5% w/v sodium chloride, +2% w/v agar for solid medium), supplemented with 100μg/ml ampicillin as required.

4.5.3 Plasmids used in this study

The plasmid pUKC1501 (Figure 4.8) carrying the *SUP35* gene, cloned downstream of the *GAL1* galactose-inducible promoter of pUKC639 (Figure 4.11), was constructed as follows. pUKC609 (Figure 4.10; gift from I. Stansfield, University of Aberdeen) was cut with *Bam*H1 and dephosphorylated. The cut-vector was then re-ligated in the presence of the oligonucleotide (5' GATCCTCCATATGGAG 3') that self-anneals to yield a *Nde*I restriction site, with *Bam*HI sticky ends. The religated product, termed pUKC1500, was purified by 'Midi-Prep', according to the manufacturer's instructions (Qiagen, UK) and digested with *Nde*I to yield a 2.3kb cassette, corresponding to the *SUP35* gene. pUKC639 (Figure 4.11; gift from K.M. Jones, University of Kent) was cut with the *Nde*I and dephosphorylated, then religated in the presence of the *SUP35 Nde*I cassette to yield pUKC1501 (as determined by restriction digest analysis; Figure 4.9). Restriction digests were performed using Boehrinhger Mannheim restriction enzymes and standard protocols (Sambrook *et al.*, 1989).

Yeast were transformed with plasmid DNA, purified by 'Mini-Prep' (Qiagen, UK), by heat shock (42°C, 1½min) following washing with lithium acetate (Geitz *et al.*, 1995). Yeast transformants were selected by growth on solid minimal medium, minus leucine.

E.coli strains were transformed with plasmid DNA, purified by 'Mini-Prep' (Qiagen, UK), by heat shock (42°C, 1½min) following washing with cold 50mM calcium chloride. Transformants were isolated by growth on solid LB medium supplemented with ampicillin (100 μg/ml).

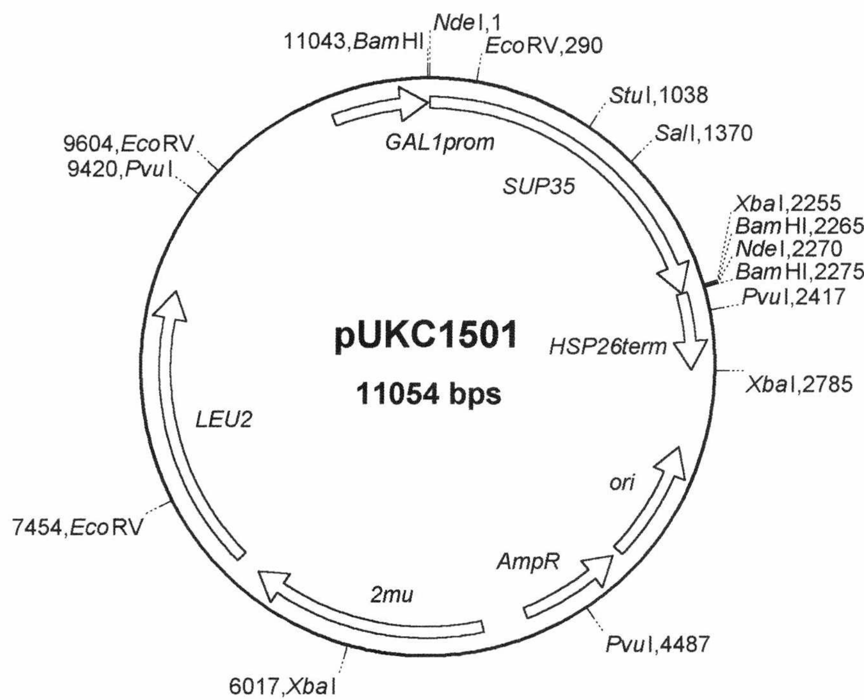


Figure 4.8 **Plasmid map of pUKC1501.** Plasmid pUKC1501 is derived from the vector pUKC639 (K.M. Jones, unpublished; see Figure 4.11). The *SUP35* gene was isolated as an *Nde*I cassette after the introduction of a second *Nde*I site at the *Bam*HI site of pUKC609 (as described in section 4.5.3) and cloned downstream of the *GAL1* promoter and upstream of the *HSP26* terminator of pUKC639.

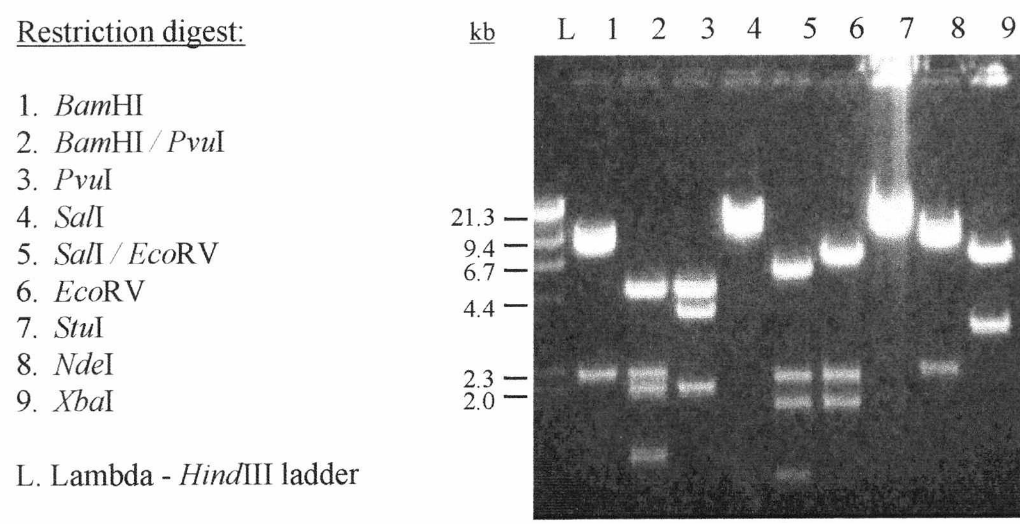


Figure 4.9 **Restriction digest of pUKC1501.** The construction of pUKC1501 was verified by an array of restriction digests. The digests yield the predicted products.

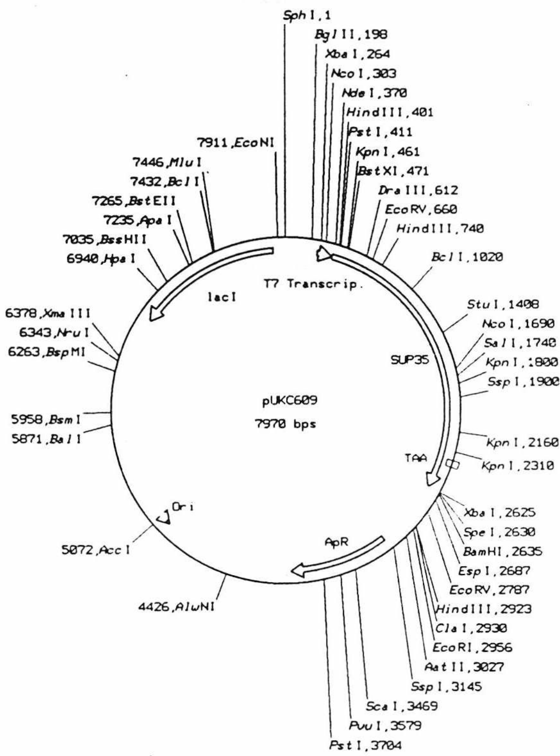


Figure 4.10 Plasmid map of pUKC609. Plasmid pUKC609 encompasses the *SUP35* gene of *Saccharomyces cerevisiae* cloned as a *Bam*HI-*Nde*I cassette into pET19B (Novagen). pUKC609 was constructed by I. Stansfield, University of Aberdeen.

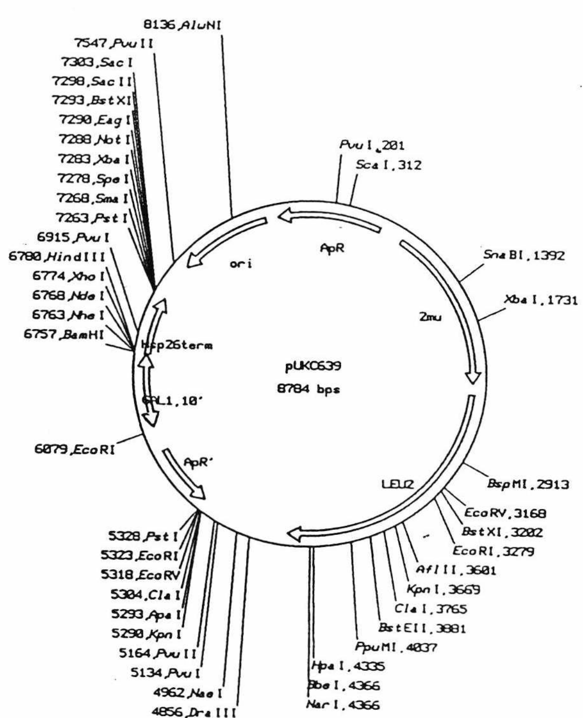


Figure 4.11 Plasmid map of pUKC639. Plasmid pUKC639 is derived from the yeast shuttle vector pRS425, with multiple cloning site and the *HSP26* terminator were introduced downstream of the *GAL1* promoter. pUKC639 was constructed by K.M. Jones, University of Kent.

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4.5.4 Sedimentation analysis of Sup35p

Yeast transformants were grown in liquid minimal (glucose) medium at 30°C, for approximately two days to generate starter cultures (final OD_{600} ~2). Yeast strains were then diluted 100-fold into galactose induction medium and grown for a further 24hrs. Culture aliquots were harvested and adjusted, with water, to the equivalent of 15ml at OD_{600} 0.5. Cells were then pelleted and lysed with glass beads at 4°C, in 500μl buffer (25mM Tris-HCl pH 8, 150mM NaCl, 5mM EDTA, 5mM PMSF, Complete Protease Inhibitor Mix [Boehringer Mannheim, 1 tablet per 5ml buffer]). Lysates were then clarified by centrifugation for 10mins at 13000 x g, 4°C and stored on ice. Samples of lysate and clarified lysate were boiled for 10mins, after the addition of 5 x SDS-PAGE sample buffer (2.5mL glycerol, 1.25mL 20% v/v SDS, 0.25mL 2-mercaptoethanol, 1.25mL 0.5M Tris-HCl pH 6.8). Samples were cooled on ice and then spun for 5 mins at 13000 x g, room temperature. Protein samples were analysed using 10% SDS-polyacrylamide gels and electrophoretically transferred to nitrocellulose for Western blot analysis, employing polyclonal sera raised against yeast Sup35p expressed in *E.coli* (as described in Stansfield *et al.*, 1995a).

4.5.5 Isoelectric focussing of Sup35p

Culture aliquots were harvested and lysed as described in section 4.5.4 above. Clarified lysates were then diluted 3-fold into IEF sample buffer (Novex, UK) before application to a pre-cast IEF gel, pH 3-10 (Cat. No. LC5311, Novex, UK). Gels were developed according to manufacturers instructions, by applying 100V 1hr, 200V 1hr and 500V 30min. The gel was soaked for 15min in a cold solution of 2% SDS, before being transferred to nitrocellulose for 1hr at 10V. Western blotting was then performed as described in section 4.5.4 above.

Chapter V

Discussion

Chapter

V

Discussion

5.1 Summary

Nearly two decades after the term 'prion' was introduced (Prusiner, 1982), the existence of self-replicating proteins has largely been accepted. The prion conformer of a cellular protein can be both the mechanism of disease in mammals (reviewed in Horwich & Weissman, 1997; Cohen & Prusiner, 1998) and a mode of inheritance in fungi (reviewed in Tuite & Lindquist, 1996; Wickner *et al.*, 1996; Coustou *et al.*, 1997; Lindquist, 1997). This study has focussed upon the yeast prion-like determinant *[PSI$^+$]* and its allosuppressor (i.e. translation termination deficient) phenotype. It has been demonstrated that *[PSI$^+$]*-induced allosuppression can confer a phenotypic advantage to yeast, namely that of enhanced stress tolerance. Furthermore, the prion-like behaviour of the *[PSI$^+$]*-determinant, namely Sup35p, was shown to provide a mechanism by which translation termination efficiency can be regulated, in response to environmental stress. These observations suggest that prions are not simply selfish proteins, thereby representing the protein equivalent of transposable elements and that the occurrence of prion proteins might be widespread, since they afford a novel mechanism of regulation and inheritance (Coustou *et al.*, 1997; Lindquist, 1997).

The elimination of the *[PSI$^+$]* prion from yeast that occurs upon growth in the presence of guanidine hydrochloride (GuHCl) has also been studied. The data demonstrate that GuHCl blocks a critical step in the propagation of the yeast prion but does not, as previously thought (Chernoff *et al.*, 1995a), promote the resolubilisation of pre-existing prion polymers (see section 3.4 above). This study of *[PSI$^+$]* 'curing' has led us to investigate an ill-defined aspect of prion biology, previously termed 'secondary nucleation' (Orgel, 1996). GuHCl appears to block the generation of nascent prion seeds (i.e. secondary nucleation). It was demonstrated that the average number of *[PSI$^+$]* seeds within a cell is ~60 and that this value appears to be constant between different strains of yeast. The implications of this are discussed below (see section 5.2.2 below).

5.2 Secondary nucleation and polymerisation

As described in Chapter 1, nucleation-dependant polymerisation (NDP; reviewed in Jarrett & Lansbury, 1993) serves as a good model for prion protein behaviour, both *in vivo* and *in vitro* (Harper & Lansbury, 1997; Lansbury, 1997b). Within the framework of the NDP model, prion seeds are polymeric nuclei that support the self-assembly of prion proteins into amyloid fibrils. The rate-limiting step in the polymerisation process is proposed to be the assembly of several monomers into a stable nucleus, an event termed 'primary (1°) nucleation'. The pathogenesis of prions is believed to be a reflection of two principal properties. First, the ability of a prion seed to corrupt the cellular protein, to the same prion conformer and second, the ability of a pre-formed prion nucleus to 'seed' rapid polymerisation upon introduction to a system, thereby circumventing the slow process of 1° nucleation. In itself, the accelerated propagation of the aberrant prion conformer is insufficient for infectivity. To be infectious, there must exist a mechanism by which a growing polymer (i.e. seed) can generate daughter nuclei, which is termed secondary nucleation (Orgel, 1996).

Ultimately, a block in either the self-assembly of the Sup35p prion conformer (i.e. growth) or an inhibition of seed proliferation (i.e. 2° nucleation) would result in the elimination of the *[PSI⁺]* determinant. However, amyloid growth and 2° nucleation are two distinct processes. Much recent research has focussed upon the self-assembly (i.e. growth) of Sup35p into amyloid fibres (Glover *et al.*, 1997; King *et al.*, 1997; DePace *et al.*, 1998). Such studies will undoubtedly continue to reveal much about *[PSI⁺]* and the properties of Sup35p (see section 5.2.3 below) but as yet they have provided little, if any, information regarding seed replication. Possibly the most important question that remains to be answered is what is the essential role of the stress protein Hsp104p in *[PSI⁺]* maintenance? Whilst it has been suggested that Hsp104p breaks-up Sup35p polymers *in vivo* (Paushkin *et al.*, 1996) thereby mediating 2° nucleation, others have suggested that Hsp104p directly catalyses the refolding of Sup35p to ensure the propagation of the prion conformer (Chernoff *et al.*, 1995a; Patino *et al.*, 1996).

5.2.1 The role of Hsp104p in *[PSI⁺]* maintenance

Hsp104p is essential for *[PSI⁺]* maintenance (Chernoff *et al.*, 1995a). The general role of Hsp104p *in vivo* seems to be that of a 'disaggregase', in that it acts to promote the rescue of stress denatured proteins from large aggregates and to facilitate their refolding to a native, functional conformation (Parsell *et al.*, 1994a; Glover & Lindquist, 1998). Hsp104p achieves this in an ATP-dependent manner (Parsell *et al.*, 1991, 1994b) and by acting in concert with two other protein chaperones, namely Ssa1p [otherwise called Hsp70] and Ydj1p [otherwise called Hsp40] (Glover & Lindquist, 1998). It has been suggested that Hsp104p 'transiently disrupts non-covalent intermolecular contacts between aggregated species' and in doing so, makes them accessible for refolding by other chaperones (Glover & Lindquist, 1998). The disruption of oligomeric Sup35p by Hsp104p is an attractive model for the 2° nucleation of *[PSI⁺]* 'amyloid' *in vivo* (Paushkin *et al.*, 1996; Glover & Lindquist, 1998).

Whilst it still remains unproven that Sup35p forms amyloid *in vivo,* the *in vitro* formation of Sup35p amyloid fibrils has been demonstrated by three independent laboratories (Glover *et al.*, 1997; King *et al,* 1997; DePace *et al.*, 1998). Glover *et al.* (1997) reported that the self-assembly of Sup35p into amyloid fibrils occurred over a prolonged period [approximately 1-3 days]. However, by simply rotating the solution of Sup35p, fibril formation was accelerated to completion within approximately 3hrs in unseeded reactions and approximately 1hr in seeded reactions (DePace *et al.*, 1998). With the doubling time of a yeast cell being approximately 2hrs, these data support the hypothesis that a growing 'amyloid' polymer undergoes continuous fragmentation *in vivo* (DePace *et al.*, 1998). Clearly, such a process could be achieved by the action of a factor such as Hsp104p.

Schirmer & Lindquist (1997) have studied the interaction of Hsp104p and Sup35p *in vitro.* On the basis of a shift from a predicted circular dichroism (CD) spectrum and loss of ATPase activity, it was concluded that Sup35p and Hsp104p do interact in a specific manner. This 'specific' interaction was coupled with a structural change within Hsp104p that eliminated its ATPase activity (Schirmer & Lindquist, 1997). Moreover, the specific

in vitro interaction between Sup35p and Hsp104p did not require the co-chaperones Ssa1p or Ydj1p and was ATP-independent. *In vivo,* the ATPase activity of Hsp104p is essential for *[PSI$^+$]*-maintenance, as demonstrated by the dominant *Psi No More* phenotype of the *hsp104-KT218,620* allele, which has both nucleotide-binding (NBD) sites inactivated (Chernoff *et al.*, 1995a). Intriguingly, it has also been demonstrated that Hsp104p could, in the presence of urea-denatured PrP^{Sc}, accelerate the conversion of PrP^{C} to a proteinase K-resistant form (DebBurman *et al.*, 1997). Again, the 'action' of Hsp104p did not require Ssa1p or Yjd1p and was ATP-independent. Remarkably, the converting activity of the two NBD mutants (K218T & K620T) was almost as efficient as the wild type protein (DebBurman *et al.*, 1997). Glover & Lindquist (1997) have suggested that 'the effect of Hsp104p on its targets may be quite different in the absence of its chaperone co-factors'. Possibly, Hsp104p could elicit the re-exposure of self-assembling interfaces or the partial unfolding of either native Sup35p or its prion conformer, thereby directly catalysing the propagation of the prion conformer (Chernoff *et al.*, 1995a; Tuite & Lindquist, 1996; Glover & Lindquist, 1998). Indeed, it has been argued that the interaction of a *[PSI$^+$]* heritable aggregate and nascent Sup35p is governed by a specific interaction with Hsp104p (Patino *et al.*, 1996).

The role of Hsp104p in the propagation of *[PSI$^+$]* might be revealed, using the *in vitro* conversion reaction of Ter-Avanesyan and colleagues (Paushkin *et al.*, 1997a). Essentially, the conversion reaction follows the fate of soluble Sup35p in a lysate prepared from a *[psi$^-$]* strain. Conversion is initiated by the addition of a highly enriched fraction, containing aggregated Sup35p isolated from a *[PSI$^+$]* strain. Upon incubation, the Sup35p originally present in the *[psi$^-$]* lysate is converted to an aggregated state. Furthermore, these aggregates are themselves able to 'seed' further rounds of conversion, thereby demonstrating the propagation of the prion conformer (Paushkin *et al.*, 1997a). We propose that the *in vitro* conversion of Sup35p be attempted, using a lysate prepared from a *HSP104* disrupted strain (and therefore *[psi$^-$]*). If successive conversions were achieved then it would demonstrate that Hsp104p is not required for the generation of the prion conformer and that Hsp104p serves as a vehicle of heritability (i.e. its action is required for 2° nucleation).

5.2.2 Prion seeds and the inheritance of *[PSI⁺]*

A 'segregational' model has been proposed that best explains the properties of the GuHCl-induced elimination of the *[PSI⁺]* determinant (see Chapter 3). The addition of GuHCl to a dividing yeast culture elicits the total inhibition of 2° nucleation and the subsequent dilution of *[PSI⁺]* seeds within a cell. In the simplest scheme, a typical *[PSI⁺]* cell initially bears ~60 seeds but this number is decreased 2-fold every generation, as the seeds are distributed at random between parent and daughter cell. The fact that these seeds are cytoplasmic seems to be irrefutable, since *[PSI⁺]* can be 'transmitted' by cytoduction (i.e. mating in the absence of nuclear fusion; Tuite *et al.*, 1978). However, it has been suggested that the seeds might not be 'free' in the cytoplasm (B.S. Cox, *pers. comm.)* and that the random segregation might actually reflect a 'cryptic' directed segregation mechanism (L.W. Ruddock, *pers. comm.*).

The recurrence of the same figure (i.e. approximately 60) for the average number of *[PSI⁺]* seeds, within a haploid cell, raises several interesting questions. For example, do the opposing processes of Sup35p self-assembly and 'clearance' of protein aggregates from the yeast limit this number? It appears that this is not the case: Hsp104p is known to promote the clearance of protein aggregates from yeast *in vivo* (Parsell *et al.,* 1994a) and Chernoff *et al.* (1995a) have demonstrated that *[PSI⁺]* is extremely sensitive to changes in levels of Hsp104p (see also Chapter 2, section 2.3.5). However, the rate of GuHCl-induced seed elimination (expressed as a function of generations) is not altered by the presence of ethanol (see Chapter 3, section 3.3.5). Ethanol induces the elevated expression of Hsp104p (Sanchez *et al.,* 1992) and would therefore be expected to accelerate the clearance of *[PSI⁺]* seeds. This is not observed demonstrating that the average seed number is the same, regardless of the level of Hsp104p expression.

If a 'kinetic' barrier does not limit the average number of *[PSI⁺]* seeds within a yeast cell then perhaps there is a physical limitation. It has been suggested that i*n vivo,* Sup35p is associated with the cell cytoskeleton (see Chapter 3, section 3.4). Perhaps a cellular structure serves as an anchorage point for *[PSI⁺]* seeds and mediates the segregation of seeds between parent and daughter cells upon cell division. The number of these cellular

structures within a cell might provide a physical limitation to the number of *[PSI⁺]* seeds per cell. Hypothetically, *[PSI⁺]* seeds might be associated with spindle fibres that mediate chromosomal segregation. This model is consistent with all of the aspects of GuHCl-induced curing so far described. Segregation of seeds would appear to be random since fewer seeds become distributed between the same number of putative anchorage sites within a cell and yet there is an equal probability that an individual seed is lost or retained by the parent cell. However, seed segregation would be directed since the physical association of a prion seed with a cell structure may be a prerequisite of *[PSI⁺]* maintenance (and indeed curing). Cell division is required for curing to permit the proliferation of anchorage sites and the subsequent dilution of seeds from individual (i.e. parent) cells.

A simple test of this hypothesis is to determine whether the curing profile of a *[PSI⁺]* diploid, triploid or tetraploid differs from that of the haploid. We predict that the average number of seeds within each of these cell types would be ~120, ~180 and ~240 respectively, on the assumption that the number of putative anchorage sites and ploidy are related. The curing of these variants should exhibit a longer segregational lag (since the average number of seeds is predicted to be greater) and the lag will exceed that of the haploid strain by ~1, 2 and 3 generation times respectively.

5.2.3 Self-assembly of Sup35p

The fate of Sup35p has been followed *in vivo*, using a hybrid protein encompassing the N-terminal PFD of Sup35p fused to green fluorescent protein [GFP] (Patino *et al.*, 1996). Upon transient expression in a *[PSI⁺]* strain, nascent tagged protein is rapidly sequestered into intense fluorescent foci thereby demonstrating that, unlike the post-translational conversion of PrP^C to PrP^{Sc} (Butler *et al.*, 1988; Caughey *et al.*, 1989), the corruption of Sup35p by a *[PSI⁺]* seed is a co-translational event. Interestingly, the GFP-Sup35p gives rise to a few very intense foci, approximately 3 per cell, in a *[PSI⁺]* strain (Patino *et al.*, 1996). These foci cannot represent *[PSI⁺]* seeds, since their numbers are far too few (i.e. much less than sixty;) to permit a curing profile that exhibits a segregational lag of several generations (see Chapter 3).

However, the fluorescent foci of GFP-Sup35p observed in a *[PSI⁺]* strain do reflect the intracellular deposition of prion protein 'amyloid' and raise the issue of the relationship between prion protein aggregation and infectivity. As defined by Orgel (1996), prion strains might exhibit different properties as a result of varying abilities to polymerise or produce new seeds. We suggest that strong and weak variants of *[PSI⁺]* might be explained in such a way. These prion 'strains' differ with respect to mitotic stability and suppression effects (Derkatch *et al.*, 1996). Weak *[PSI⁺]* strains are mitotically unstable (i.e. have an elevated frequency of spontaneous *[psi⁻]* appearance), which might reflect an inherent decrease of 2° nucleation. The lower the average number of *[PSI⁺]* seeds within a cell, the higher the chance of a seed-free (i.e. *[psi⁻]*) cell being produced upon mitotic division. Similarly, the decreased number of *[PSI⁺]* seeds within a cell would not be expected to support the efficient 'sequestration' of Sup35p to the prion conformer, resulting in a weak allosuppressor phenotype (i.e. only a modest increase in translation termination inefficiency). The converse argument can be applied to the strong *[PSI⁺]* phenotype, which is mitotically stable and exhibits strong allosuppression.

The recent *in vitro* propagation of the Sup35p prion conformer has also afforded some intriguing explanations for other aspects the *[PSI⁺]* phenomenon (Paushkin *et al.*,1997a; DePace *et al.*, 1998; Kochneva-Pervukhova *et al.*, 1998). Mutant Sup35p that give rise to an antisuppressor phenotype (i.e. efficient translation termination) *in vivo*, exhibit decreased rates of aggregation *in vitro* (DePace *et al.*, 1998). This may result either from an inability to form stable prion seeds, which are capable of supporting polymerisation, or from an inherent incapacity to self-assemble (i.e. grow) into stable 'amyloid'. Kochneva-Pervukhova *et al.* (1998) have suggested that the *PNM2* allele, which encodes a G58D substitution in Sup35p, cannot support *[PSI⁺]* under normal cellular conditions because the mutant protein exhibits a decreased rate (~2-fold) of prion conversion. By overexpressing the mutant protein (i.e. Sup35p[G58D]), it was shown that *[PSI⁺]* could be supported (Kochneva-Pervukhova *et al.*, 1998). Similarly, it is predicted that by reducing the growth rate of a yeast strain (e.g. by the addition of ethanol) it would be possible to support *[PSI⁺]* with wild type levels of the *PNM2* mutant protein. It is apparent that the study of the yeast *[PSI⁺]* phenotype *in vivo* and the formation of Sup35p amyloid *in vitro* comprise a unique system for developing an understanding of the behaviour of prion proteins and prion phenomena in general.

5.3 Sup35p: a paradigm for mammalian prion protein?

The prion hypothesis (Prusiner, 1982) has been applied to describe the 'phenotypic' properties of two apparently unrelated proteins, namely Sup35p and PrP (see Chapter 1). These proteins are not homologous with respect to primary sequence or function, but do possess a common N-terminal structure that encompasses a series of peptide repeats (see Chapter 1, section 1.4.1 & Chapter 4, Figure 4.7). Whilst this region is essential for the propagation of the Sup35p prion conformer and the *[PSI⁺]* phenotype (Ter-Avanesyan *et al.*, 1994), the N-terminal domain of PrP is non-essential for mammalian prion disease (Fischer *et al.*, 1996). It is proposed that Sup35p and PrP are both members of a large family of amyloidogenic proteins. The amyloid proteins share little (if any) sequence homology but exhibit a common propensity to fold and assemble, apparently via a common mechanism (i.e. nucleation dependent polymerisation), into a cross-β fibrilar quaternary structure (reviewed in Wetzel, 1996; Horwich & Weissman, 1997; Kelly, 1997; Lansbury, 1997a, b).

The aberrant structural properties of both the Sup35p and PrP prion conformer confers resistance to proteolytic degradation (McKinley *et al.*, 1983; Paushkin *et al.*, 1996). Similarly, the same non-native folding of these proteins elicits their self-assembly into 'amyloid'. However, unlike the extracellular deposition of PrP (Merz *et al.*, 1981; Hope *et al.*, 1988b), Sup35p aggregates are intracellular (Patino *et al.*, 1996; Paushkin *et al.*, 1996). Another critical difference is that the conversion of the 'cellular' mammalian protein, to the prion conformer, is a post-translational event (Borchelt *et al.*, 1990; Caughey & Raymond, 1991), whereas the corruption of yeast Sup35p is co-translational (Patino *et al.*, 1996). It has been postulated that the 'rapid' conversion time, as determined for nascent Sup35p *in vitro* (approximately 2hrs; DePace *et al.*,1997; Paushkin *et al*, 1997a) permits *[PSI⁺]* maintenance, since the time of prion replication is less than that of one cell generation. The conversion of PrP would not presumably have such a 'requirement' since prion propagation occurs within non-dividing cells and might therefore be expected to proceed at a much slower rate (Prusiner, 1982). However, Borchelt *et al.* (1992) demonstrated that whilst the conversion of radioactive PrP^C, in cultured cells, did occur post-translationally (i.e. radiolabelled PrP^{Sc} was formed several

hours after synthesis), the conversion process was almost complete within 10hrs. This might suggest that the pathogenic manifestation of PrP^{Sc} (i.e. the duration of the incubation period) is not limited by the ability of the prion conformer to propagate, but by other factors such as 'clearance' from the cell or by a physical barrier that precludes transmission. For example, the spread of the mammalian prion 'phenotype' (i.e. prion disease) requires the 'transmission' of PrP^{Sc} from the host cell to a neighbouring uninfected cell. In *S. cerevisiae,* the transmission of phenotype occurs readily as the self-replicating prion is segregated between parent and daughter cells upon mitotic division.

Chernoff *et al.* (1995a) demonstrated that the propagation of the Sup35p prion exhibits an absolute requirement for the action of an ancillary factor, namely Hsp104p. Whilst there is no direct evidence for such an interaction, it has been suggested that the conversion of PrP^{C} to PrP^{Sc} might also be heat shock protein dependant (Telling *et al.*, 1995). Indeed there is precedent for such a relationship *in vivo* between the Alzheimer's amyloid β-peptide and apolipoprotein E (Evans *et al.*, 1995; Golabek *et al.*, 1996). The yeast prion determinant *[PSI⁺]* can be eliminated both by the overexpression of Hsp104p or by the loss of its activity (Chernoff *et al.*, 1995a). It has been suggested that if PrP^{Sc} formation is dependant upon the action of a second protein, for example a protein chaperone, this might afford an opportunity for therapeutic intervention (Telling *et al.*, 1995; Kenward *et al.*, 1996). This study has investigated the inhibition of yeast prion replication and highlighted the distinction between amyloid growth and prion seed replication (see Chapter 4). Just as GuHCl inhibits *[PSI⁺]* propagation in yeast (Tuite *et al.*, 1981a), various compounds have been described as inhibitors of PrP^{Sc} formation (Tatzelt *et al.*,1996; Caspi *et al.*, 1998). However, as a note of caution, it should be appreciated that unlike *[PSI⁺]*, which appears to utilise the 'fortuitous' action of Hsp104p for its own replication, PrP^{Sc} propagation (and hence disease) might be 'limited' by a slow rate of secondary nucleation (Orgel, 1996). The disruption of PrP^{Sc} 'amyloid' *in vivo,* either by chemical means or by the elevated induction of stress proteins, might only serve to accelerate prion seed replication (i.e. secondary nucleation) and ultimately the pathology of the prion disease.

In conclusion, it is suggested that yeast prion protein Sup35p is not a suitable model for the mammalian prion protein PrP itself. However, the study of the yeast protein-based

phenotype *[PSI$^+$]* and the interaction of its protein determinant (i.e. Sup35p) with cellular factors promises to further our understanding of the behaviour of amyloidogenic proteins *in vivo*. Undoubtedly, the study of amyloidogenic proteins will benefit from the relatively rapid growth of *Saccharomyces cerevisiae* and the 'awesome power of yeast genetics'. The first lesson to come from the study of prions in fungi is that amyloidogenic proteins are not always associated with disease. The prion-like properties of certain yeast proteins serve as a means of inheritance (reviewed in Tuite & Linquist, 1996; Wickner *et al.*, 1996) and may afford a novel means by which cellular processes can be regulated.

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Translation termination efficiency can be regulated in *Saccharomyces cerevisiae* by environmental stress through a prion-mediated mechanism

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[PSI+] is a protein-based heritable phenotype of the yeast *Saccharomyces cerevisiae* which reflects the prion-like behaviour of the endogenous Sup35p protein release factor. [PSI+] strains exhibit a marked decrease in translation termination efficiency, which permits decoding of translation termination signals and, presumably, the production of abnormally extended polypeptides. We have examined whether the [PSI+]-induced expression of such an altered proteome might confer some selective growth advantage over [psi⁻] strains. Although otherwise isogenic [PSI+] and [psi⁻] strains show no difference in growth rates under normal laboratory conditions, we demonstrate that [PSI+] strains do exhibit enhanced tolerance to heat and chemical stress, compared with [psi⁻] strains. Moreover, we also show that the prion-like determinant [PSI+] is able to regulate translation termination efficiency in response to environmental stress, since growth in the presence of ethanol results in a transient increase in the efficiency of translation termination and a loss of the [PSI+] phenotype. We present a model to describe the prion-mediated regulation of translation termination efficiency and discuss its implications in relation to the potential physiological role of prions in *S.cerevisiae* and other fungi.

Keywords: environmental stress/phenotype/prion/yeast (*Saccharomyces cerevisiae*)

Introduction

It is some 30 years since the extrachromosomal determinant *[PSI+]* was first described in the yeast *Saccharomyces cerevisiae* as a modifier of nonsense suppression (Cox, 1965). The *[PSI+]* determinant confers a dominant allosuppressor phenotype, with *[PSI+]* strains exhibiting a marked increase in the readthrough of mutant translation termination codons (Cox *et al.*, 1988). In 1994, Wickner proposed that *[PSI+]* may represent an example of protein-based inheritance, whereby the heritable element behaves like an endogenous 'prion-like' determinant (Wickner, 1994). The term prion was first used to describe the proteinacious agent thought to be solely responsible for the family of neurodegenerative diseases, known as the transmissible spongiform encephalopathies (Griffith, 1967; Prusiner, 1982; Weissman, 1996; Horwich and Weissman, 1997).

In yeast, the *[PSI+]* factor is a product of the *SUP35* gene (Chernoff *et al.*, 1993; Doel *et al.*, 1994; Ter-Avanesyan *et al.*, 1994) which encodes eRF3 (Sup35p), an essential eukaryotic polypeptide release factor. Eukaryote translation termination is mediated by a soluble cytoplasmic complex, which encompasses eRF3 and at least one other factor, namely eRF1 [Sup45p] (Stansfield *et al.*, 1995a; Zhouravleva *et al.*, 1995). As well as folding into its native structure, Sup35p is believed to be capable of adopting a second aberrant conformation, which manifests as the prion-associated phenotype (Chernoff *et al.*, 1995; Paushkin *et al.*, 1996; Tuite and Lindquist, 1996). In *[PSI+]* strains, Sup35p is present both as a soluble factor and as large intracellular aggregates, resulting from the propensity of the prion conformer to coalesce (Patino *et al.*, 1996; Paushkin *et al.*, 1996). The resulting intracellular depletion of soluble termination factors facilitates the decoding of termination signals by mutant nonsense suppressor tRNAs, or by near-cognate aminoacyl-tRNAs, giving rise to the prion-associated allosuppressor phenotype. Intriguingly, both Sup35p and the mammalian prion protein (PrP) possess a conserved N-terminal domain, comprising several imperfect glutamine-rich repeats (Oesch *et al.*, 1985; Kushnirov *et al.*, 1988). Whilst this motif is not a prerequisite for the aetiology of the PrP-associated diseases (Fischer *et al.*, 1996), the N-terminal domain of Sup35p is essential for the maintenance of *[PSI+]* (Ter-Avanesyan *et al.*, 1994). Although the prion-inducing domain of Sup35p is essential for neither translation termination nor viability, it is nonetheless conserved and expressed in *S.cerevisiae* (Ter-Avanesyan *et al.*, 1993; Doel *et al.*, 1994).

Whilst no detrimental phenotype has been associated with *[PSI+]*, neither has a benefit been ascribed to the presence of the prion-inducing domain of Sup35p and its prion-associated phenotype. One possible scenario is that the conservation of this prion-associated domain may represent a novel means of environmental adaptation, whereby *[PSI+]*-induced allosuppression permits decoding of translation termination signals, resulting in an altered pattern of gene expression and the 'profitable' generation of abnormally extended polypeptides (Lindquist, 1997). In an effort to identify such a phenotypic advantage, the stress tolerance of otherwise isogenic *[PSI+]* and *[psi⁻]* strains was compared. A strain bearing a nuclear allosuppresor mutation of the *SUP35* gene (*sal3-4*) was also studied, since this mutation elicits a similar translation termination deficiency as the *[PSI+]* element, without the associated polymerization of eRF3. Using the *ade2-1/SUQ5* (Cox, 1965) and *lacZ*-'readthrough' reporter systems (Firoozan *et al.*, 1991; Stansfield *et al.*, 1995b), it was possible to study allosuppression in both *[PSI+]* and *sal3-4* strains, when grown under both 'resting' and 'stressed' conditions. Our findings suggest that *[PSI+]*

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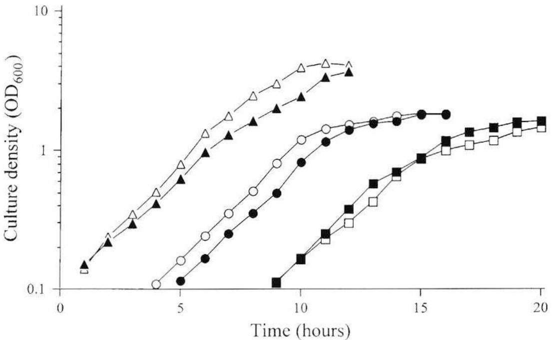

Fig. 1. *[PSI⁺]* and *[psi⁻]* variants of *S.cerevisiae* strains show no difference in growth under resting conditions. The exponential growth of the *[PSI⁺]* (filled symbols) and *[psi⁻]* (empty symbols) variants of three strains was compared. Strains (●○) BSC 783/4a, (▲△) MT 766/12a and (■□) BSC 772/9d were grown in minimal medium at 30°C.

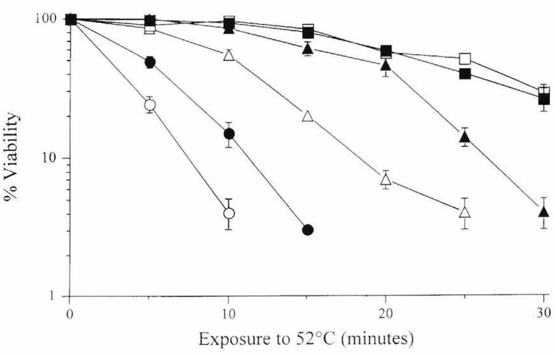

Fig. 2. *[PSI⁺]* strains exhibit an enhanced tolerance to thermal stress. The viability of *[PSI⁺]* (filled symbols) and *[psi⁻]* (empty symbols) variants of three strains (●○) BSC 783/4a, (▲△) MT 766/12a and (■□) BSC 772/9d was monitored, upon exposure to a lethal temperature of 52°C, following a 1 h pre-treatment at 37°C. At regular intervals, aliquots were removed from the cultures at 52°C and put on ice. Samples were diluted and then spread in triplicate onto solid YEPD medium to generate a viable count. Each sample gave rise to ~400 cells per plate, and the error bars illustrate the variation between three viable counts per sample.

strains do exhibit a phenotypic difference from *[psi⁻]* strains, namely enhanced tolerance to physical and chemical stresses, and we conclude that the prion-like behaviour of Sup35p may facilitate the adaptation of yeast to new growth environments.

Results

[PSI⁺] does not influence growth rate

The only phenotypic difference between *[PSI⁺]* and *[psi⁻]* strains so far described is the enhanced efficiency of nonsense suppression (Cox, 1965) and the elevation of stop codon readthrough in quantitative assays (Firoozan *et al.*, 1991) in *[PSI⁺]* cells. Given that >90% of the underlying Sup35p protein—an essential translation termination factor—is present as non-functional aggregates in *[PSI⁺]* strains (Patino *et al.*, 1996; Paushkin *et al.*, 1996), then one might expect *[PSI⁺]* strains to have an associated slow growth phenotype. Three *[PSI⁺]* strains were selected on the basis of their difference in genetic background and for the presence of the *ade2-1/SUQ5* reporter system (Cox, 1965). The *[psi⁻]* variant of each strain was induced by growth in the presence of 2.5 mM guanidine hydrochloride, a compound which induces a *[PSI⁺]* to *[psi⁻]* reversion with near 100% efficiency (Tuite *et al.*, 1981). The exponential growth of the three pairs of *[PSI⁺]* and *[psi⁻]* strains was compared in minimal medium at 30°C (Figure 1). As demonstrated by the identical growth profiles of the *[PSI⁺]* and *[psi⁻]* variant of each of three strains, it is clear that the prion-like determinant *[PSI⁺]* does not influence the exponential propagation of yeast in typical laboratory culture conditions, with the doubling times of each pair being essentially identical.

[PSI⁺] strains exhibit an enhanced thermotolerance

Whilst *[PSI⁺]* does not elicit any obvious change in growth rate, it was considered that the prion-associated termination deficiency might manifest as an activation of the cellular stress response. The stress response machinery acts to protect cells against the detrimental effects of changes in the extracellular environment, such as an increase in temperature or ethanol concentration. Stresses that induce the synthesis of aberrant proteins also lead to the overexpression of a particular subset of proteins, namely the heat shock or stress proteins. For example, in yeast, the antibiotic paromomycin, which stimulates mistranslation on cytoplasmic ribosomes, also induces the heat shock response (Grant *et al.*, 1989). In a termination-deficient *[PSI⁺]* background, the production of abnormal C-terminally extended proteins might also be expected to trigger such a response. Alternatively, the presence of aggregated Sup35p itself might trigger the stress response.

Thermotolerance assays were used, therefore, in order to quantify any stress response conferred by the *[PSI⁺]* determinant. Strains subjected to a short period of stress or experiencing a constant stimulus of the stress response will display an enhanced viability upon exposure to a lethal temperature, reflecting an elevation of the intracellular concentration of heat shock proteins (Sanchez and Lindquist, 1990; Mager and Ferreira, 1993). Thermotolerance analysis of the three pairs of strains demonstrated that, for two of the three pairs, the *[PSI⁺]* strains exhibited a significant increase in thermotolerance, in comparison with the corresponding *[psi⁻]* variants (Figure 2). However, in the third strain examined (BSC 772/9d), no difference in thermotolerance between the *[PSI⁺]* and *[psi⁻]* variants was observed. This particular strain has a 'naturally' high degree of thermotolerance (LD_{50} of 25 min at 52°C; Figure 2) which we assume masks any effect *[PSI⁺]* may have had on thermotolerance. Given the integral relationship between *[PSI⁺]* and Hsp104p (Chernoff *et al.*, 1995), strain MT 766/12a was employed to address the role, if any, of Hsp26p in the maintenance of *[PSI⁺]* or the stress-induced prion-mediated regulation of translation termination efficiency. Clearly, this *HSP26*-disruptant strain can support *[PSI⁺]*, thereby demonstrating that Hsp26p is not essential for *[PSI⁺]* maintenance. Our data support previous studies which demonstrate that Hsp26p has little, if any, role in thermotolerance (Figure 2) (Tuite *et al.*, 1990). Whilst *[PSI⁺]* strains exhibited an enhanced thermotolerance, Western blot analyses failed to reveal any difference in the endogenous level of the heat shock proteins Hsp104, Hsp70 and Hsp26 between the *[PSI⁺]*

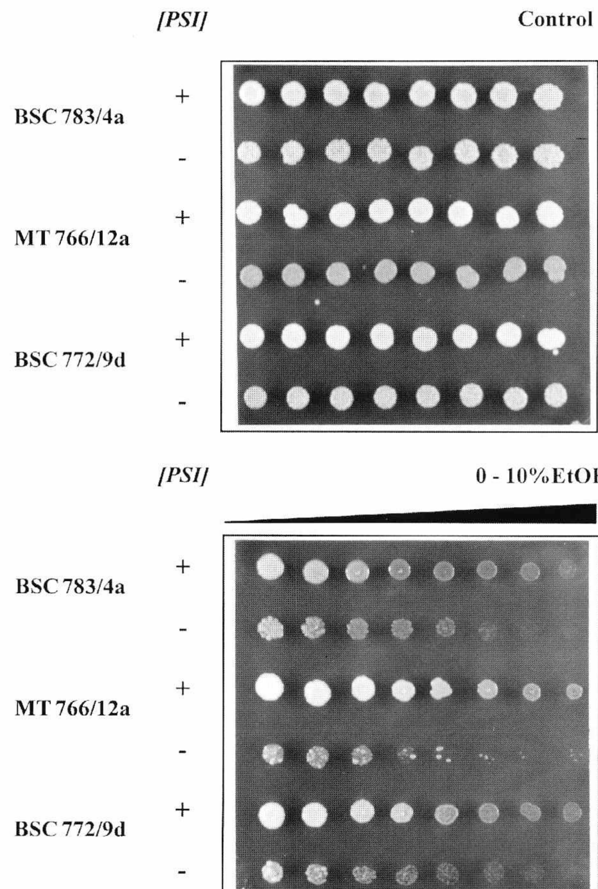

Fig. 3. *[PSI⁺]* strains exhibit an enhanced tolerance to ethanol. The stress tolerance of the *[PSI⁺]* and *[psi⁻]* variants of each of the three test strains was compared by growth upon solid YEPD medium, containing a gradient of 0–10% (v/v) ethanol. Strains suspensions were normalized with respect to cell density, and equal volumes (i.e. numbers) of cells were spotted either onto solid 1/4YEPD medium (control) or onto a plate containing a gradient of 0–10% ethanol. Plates were then incubated at 30°C for 5 days.

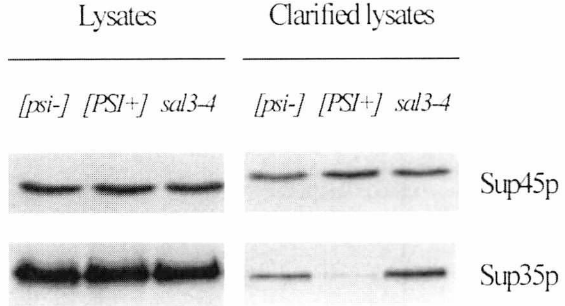

Fig. 4. The *sal3-4* mutant encodes a soluble part-functional component of the eukaryote release factor. The sedimentation properties of the two eukaryotic release factors, Sup35p and Sup45p, were determined in the *[PSI⁺]* and *[psi⁻]* variants of strain BSC 783/4c and in a mutant strain bearing the nuclear allosuppressor allele, *sal3-4*. Proteins were detected by Western blot analyses of whole-cell lysates and the soluble fraction of total cell extracts, following the clarification of lysates by centrifugation.

Table I. Quantification of termination efficiency in strains bearing the *[PSI⁺]* and *sal3-4* allosuppressor determinants, upon growth in a stress-inducing medium

	% Readthrough		
	[psi⁻]	*[PSI⁺]*	*[psi⁻] sal3-4*
0% Ethanol	4.7 ± 1.0	23.8 ± 5.7	18.5 ± 3.8
7% Ethanol	3.9 ± 0.8	7.7 ± 1.5	15.6 ± 3.0

The level of termination signal readthrough was measured in strain BSC 783/4c, using a β-galactosidase reporter system (described by Stansfield *et al.*, 1995b). Readthrough of termination codons was quantified using the plasmid pUKC817 (which carries the *lacZ* gene that bears a premature termination codon) and expressed as a proportion of control β-galactosidase levels, measured in transformants carrying the control plasmid pUKC815 (which carries the wild-type *lacZ* gene).

and *[psi⁻]* variants when grown at 25°C. Similarly, whilst an elevation of Hsp104p expression was detected upon heat shock at 37°C, no significant difference was observed between *[PSI⁺]* and *[psi⁻]* strains (unpublished data).

[PSI⁺] strains exhibit enhanced chemotolerance

Having demonstrated that *[PSI⁺]* enhances thermotolerance, we next asked whether *[PSI⁺]* enhanced tolerance to other forms of stress. *In vivo*, some components of the stress response machinery do confer tolerance to more than one form of stress. For example, Hsp104p is known to be the principal agent that confers tolerance to both heat and ethanol (Sanchez and Lindquist, 1990; Sanchez *et al.*, 1992; Piper, 1995). The growth of the *[PSI⁺]* and *[psi⁻]* variants was therefore compared on gradient agar plates which contain an increasing concentration of stress reagent in a rich growth medium. As with thermotolerance, the *[PSI⁺]* strains exhibited a greater degree of tolerance to 'chemical stress' than the *[psi⁻]* variants. The most marked enhancement of chemotolerance was observed when the strains were grown on gradients of ethanol (Figure 3). In contrast, no difference in growth was observed between the *[PSI⁺]* and *[psi⁻]* variants when grown on gradients of glycerol or salts such as sodium, potassium or magnesium chloride (unpublished data). Therefore, *[PSI⁺]* strains appear to show a higher degree of resistance to potential environmental abuses than the corresponding *[psi⁻]* strains.

Allosuppression enhances stress tolerance

To determine how the *[PSI⁺]* determinant elicits an enhanced stress tolerance, the thermo- and chemotolerant properties of a [*PSI⁺*] strain and a [*psi⁻*] strain bearing the *sal3-4* mutation were compared. The *sal3-4* mutation lies in the *SUP35* gene (Doel *et al.*, 1994), presumably within the functional C-terminal domain of the encoded eRF3, thereby leading to a termination defect not through Sup35p aggregation, but from biochemical malfunction. Western blot analysis of the *sal3-4* mutant strain, using polyclonal antiserum raised against Sup35p, confirmed that unlike the *[PSI⁺]* strains under examination, the *sal3-4* allele encodes primarily soluble Sup35p (Figure 4). The *[PSI⁺]* strain BSC 783/4c and the *sal3-4* allosuppressor mutant elicited a similar degree of termination codon readthrough (Table I) and thus are presumed to elicit the same degree of production of C-terminally extended polypeptides. Like the *[PSI⁺]* strains, the *sal3-4* mutant did not exhibit an impairment of exponential growth (unpublished data). The *sal3-4* mutant also showed

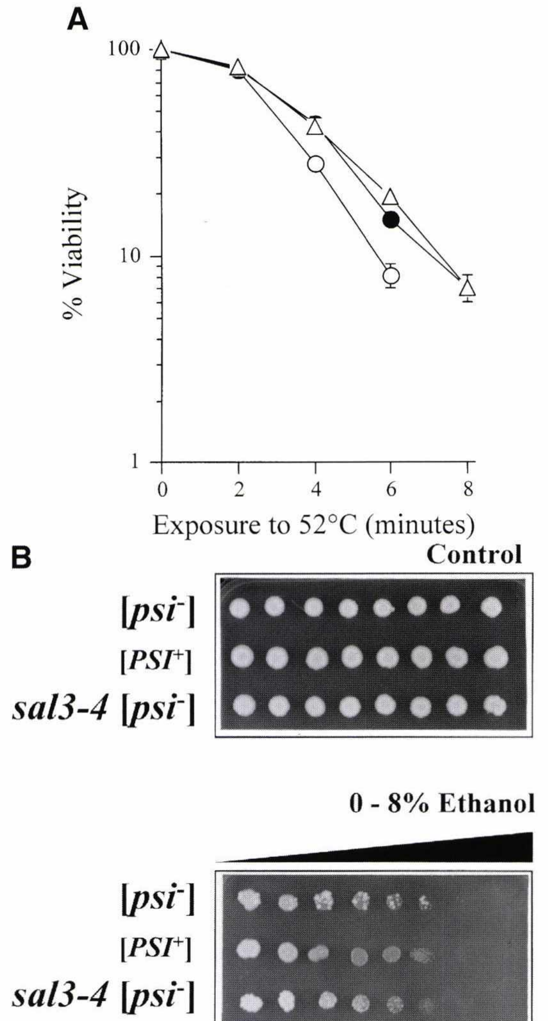

Fig. 5. The prion-like determinant *[PSI$^+$]* and the nuclear allosuppressor mutation *sal3-4* both elicit enhanced tolerance to stress. (**A**) The thermotolerance of the *[PSI$^+$]* (●) and the *[psi$^-$]* (○) variants of BSC 783/4c was compared with that of a *[psi$^-$]* variant of BSC 783/4c that bears the nuclear allosuppressor allele *sal3-4* (△). Cultures were exposed to a lethal temperature (52°C), following a 1 h pre-treatment at 37°C. At regular intervals, culture aliquots were removed from 52°C and put on ice. Samples were then diluted and spread in triplicate onto solid YEPD medium to generate a viable count. Each sample gave rise to ~400 cells per plate, and the error bars represent the variation between three viable counts per sample. (**B**) The ethanol tolerance of the BSC 783/4c variants was also compared by studying their growth on solid rich medium supplemented with ethanol. Strain suspensions were normalized with respect to cell density, and equal volumes (i.e. numbers) of cells were spotted either onto solid 1/4YEPD medium (control) or onto a plate containing a gradient of 0–8% ethanol. Plates were incubated for 5 days at 30°C.

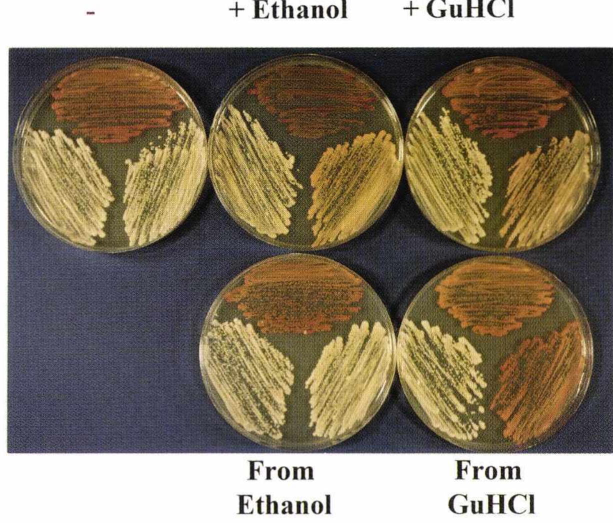

Fig. 6. Stress induces a reversible antisuppression in *[PSI$^+$]* strains, but not in strains bearing the nuclear allosuppressor mutation *sal3-4*. Top row: the variants of strain BSC 783/4c were grown on control 1/4YEPD medium (left) and medium supplemented with either 5% ethanol (middle) or 2.5 mM guanidine hydrochloride (right). Each plate: strain BSC 783/4c, clockwise from the top *[psi$^-$]*, *[PSI$^+$]* and *[psi$^-$] sal3-4*. Bottom row: variants were then re-streaked from stress-inducing media (top row) onto control 1/4YEPD medium. By virtue of the *ade2-1/SUQ5* genetic markers, variants that exhibit a translation termination deficiency (i.e. nonsense suppression) develop as white colonies, whereas strains efficient for translation termination accumulate a red pigment.

enhanced stress tolerance (Figure 5), thereby demonstrating that it is a deficiency of translation termination that is the principal trigger for an elevated intracellular stress response in *[PSI$^+$]* strains, rather than the presence of Sup35p aggregates.

[PSI$^+$] strains exhibit antisuppression when stressed

Intriguingly, *[PSI$^+$]* strains exhibited a transient loss of their allosuppressor phenotype when grown on certain 'chemical' media. That is to say, *[PSI$^+$]* strains bearing the *SUQ5/ade2-1* reporter genes gave rise to pink or red colonies (reflecting an enhanced efficiency of translation termination) when grown on stress-inducing, ethanol-containing media (Figure 6). This stress-induced 'antisuppression' was not simply 'curing' of the *[PSI$^+$]* prion (as is induced by guanidine hydrochloride; Tuite *et al.*, 1981), since the stressed *[PSI$^+$]* strains reverted to an allosuppressor phenotype upon transfer to ethanol-free media (Figure 6). Growth in the presence of the stress-inducing agent did not result in the heritable loss of the *[PSI$^+$]* determinant, despite the transient loss of the *[PSI$^+$]* phenotype. The degree of stress-induced antisuppression varied between the various *[PSI$^+$]* strains, but antisuppression was invoked readily in *[PSI$^+$]* strains by growth in the presence of ethanol (Table I). It is noteworthy that strain MT 766/12a also exhibited a transient antisuppressor phenotype when grown at 37°C (unpublished data). In contrast, a strain bearing the nuclear allosuppressor mutation *sal3-4* failed to exhibit antisuppression in response to stress (Table I, Figure 6). Thus, strains bearing the prion-like determinant *[PSI$^+$]* exhibit a reversible decrease in termination codon readthrough (i.e. antisuppression) when grown under stress which, unlike growth on guanidine hydrochloride, does not result from the elimination of the *[PSI$^+$]* determinant. Western blot analysis of Sup35p in *[PSI$^+$]* cells exposed to ethanol did not reveal a significant increase in the levels of soluble Sup35p (data not shown). This suggests that the *[PSI$^+$]* phenotype can be transiently reversed by the low level of newly synthesized Sup35p molecules which are unable to enter aggregates or, alternatively, by the release of previously synthesized Sup35p molecules from the pre-formed *[PSI$^+$]* aggregates.

Discussion

The two yeast prion elements so far described, namely *[PSI⁺]* and *[URE3]*, may both serve as regulators of different cellular physiological processes, namely translation termination and nitrogen catabolism, respectively (Cox, 1994; Wickner and Masison, 1996). Since this realization, there has been considerable speculation as to the much wider role of prions in biological systems (Patino *et al.*, 1996; Tuite and Lindquist, 1996; Wickner and Masison, 1996; Lindquist, 1997). Indeed, this notion appears to be supported by the recent description of a prion-like element, which confers heterokaryon incompatability, in the filamentous fungus *Podospora anserina* (Coustou *et al.*, 1998). If prion elements and their associated heritable traits are prevalent throughout different species, they should presumably confer some benefit to the organism carrying them. By studying the growth of *[PSI⁺]* and *[psi⁻]* yeast strains under adverse environmental conditions, we have demonstrated that the yeast prion *[PSI⁺]* does indeed confer a beneficial phenotypic difference, namely that of enhanced tolerance to environmental stress.

Both the prion-like determinant *[PSI⁺]* and the nuclear allosuppressor mutation *sal3-4* are derived from the *SUP35* gene and confer a partial translation termination defect to yeast (Table I). In this study, we have shown that the *sal3-4* mutation elicits an increase in translation termination codon readthrough, similar to that conferred by the *[PSI⁺]* determinant, and that the *sal3-4* allosuppressor mutation results in the expression of a soluble, but partially non-functional polypeptide release factor. This is different from *[PSI⁺]*-induced allosuppression that arises from the prion-like propensity of a conformer of Sup35p to aggregate, which results in the intracellular depletion of functional soluble termination factor (Paushkin *et al.*, 1996, 1997; Tuite and Lindquist, 1996). Both prion- and nuclear mutation-induced allosuppression produce an enhancement of thermo- and chemotolerance. Plausibly, the readthrough of translation termination codons elicits an enhancement of stress tolerance via a constitutive stress response, which is invoked by the presence of proteins that have misfolded C-terminal extensions. This notion has also been applied to account for the evolution of a $tRNA^{Ser}$ of *Candida albicans* which inserts serine at the leucine-encoding codon CUG (Santos *et al.*, 1996). Transformation of *S.cerevisiae* with a gene encoding this recoded tRNA generates an alternative 'mistranslated' proteome that also results in an enhancement of stress tolerance.

Rather than increasing stress tolerance by triggering a constitutive stress response, it might be suggested that *[PSI⁺]*-induced allosuppression (and indeed the same would be true of the *sal3-4* mutation) permits the production of one or more novel protein(s) which bear an extra C-terminal domain. The modified biochemical properties of such elongated polypeptides might directly enhance stress tolerance; for example, if these extended polypeptides stabilized the cell membrane. Whilst *[PSI⁺]* leads to an increased tolerance of *S.cerevisiae* to adverse environmental conditions, *[PSI⁺]* is not a regulator of the stress response. The potentially beneficial phenotype of enhanced stress tolerance is a consequence of *[PSI⁺]*-induced allosuppression. Whereas strains bearing the *sal3-4* allosuppressor mutation do not exhibit a significant change in termination efficiency in response to increased environmental stress, the prion-like element *[PSI⁺]* mediates a reversible, stress-induced increase in translation termination efficiency (Figure 6; Table I). Paradoxically, the elevated intracellular stress response which results from the prion-induced production of extended polypeptides (and manifests as an enhancement in thermotolerance) is selected against in an adverse environment since the generation of misfolded proteins is disfavoured by the prion-mediated decrease in the extent of translation termination codon readthrough.

Deletion analysis has revealed that whilst the N-terminal domain of Sup35p is essential for the maintenance of *[PSI⁺]*, it is not essential for cell viability, and thus appears to play no direct functional role in translation termination (Ter-Avanesyan *et al.*, 1993, 1994). However, this glutamine-rich domain of Sup35p has been conserved in *S.cerevisiae*, either as an indirect result of the *[PSI⁺]* phenotype or because of some as yet unidentified function. Whilst *[PSI⁺]* recently has been described as a 'laboratory-confined disease' of *S.cerevisiae* (Chernoff *et al.*, 1998), we have demonstrated that the prion element *[PSI⁺]* does not infleunce exponential growth, but does actually confer a beneficial phenotype to yeast, namely enhanced stress tolerance. Intriguingly, there is evidence to suggest that Sup35p does serve a role in eukaryotes other than that of a polypeptide release factor. For example, Sup35p has been identified as a putative cell cycle factor of *S.cerevisiae* (Kikuchi *et al.*, 1988), and recently the *Drosophila* homologue of yeast Sup35p has been shown to mediate meiotic spindle assembly (Basu *et al.*, 1998).

In *S.cerevisiae*, *[PSI⁺]* represents a novel mechanism for the inheritence of a regulated translation termination deficiency. Clearly, the lethal consequences of a nonsense mutation in an essential gene would be overcome in an allosuppressor background. *[PSI⁺]* is an omnipotent allosuppressor determinant, in that it enhances nonsense suppression of all three termination codons (Firoozan *et al.*, 1991), thus *[PSI⁺]* would be expected to offer protection against most potentially lethal nonsense mutations. One such example has been described in which a strain exhibits a *[PSI⁺]* dependence, since it bears a nonsense mutation within the essential gene *HSF1*, which encodes the heat shock transcription factor (Lindquist and Kim, 1996). Alternatively, *[PSI⁺]* might permit the regulated expression of abnormally extended polypeptides, which results from the extended decoding of open reading frames (ORFs) by by-passing ORF-defining termination codons. Genome analysis has revealed the existance of several potentially 'readthrough-regulated' genes (Lindquist, 1997) but, as a caution, it should be noted that translation termination is not only regulated by the interaction of release factors with termination codons. In particular, the efficiency of nonsense suppression, and hence the expression of any abnormally extended polypeptide, will also be governed by the presence and properties of endogenous nonsense suppressor tRNA and by the 'context' of the stop signal within the mRNA (Fearon *et al.*, 1994; Bonetti *et al.*, 1995). Our data suggest that *[PSI⁺]* does not promote the alternative expression of genes whose products are absolutely required for

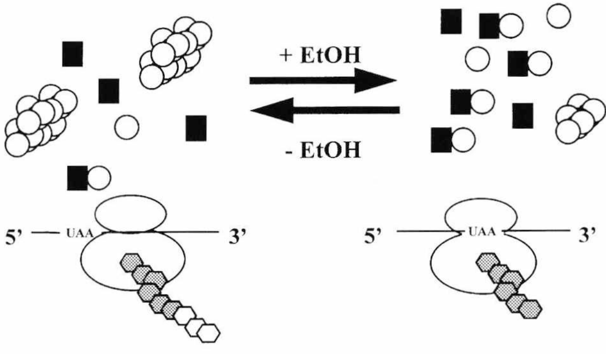

Fig. 7. A model for the prion-mediated regulation of translation termination. In a *[PSI⁺]* background, efficient translation termination is prevented by the intracellular polymerization of Sup35p and depletion of a functional eRF3 (Sup35p) (○)–eRF1 (Sup45p) (■) termination factor complex. An increase in environmental stress, such as an increase in ethanol concentration, transiently elevates the production of stress proteins (e.g. Hsp104), which mediates the partial resolubilization of Sup35p aggregates. The emergence of soluble Sup35p elicits an increase in translation termination efficiency. Upon restoration of the cell to a normal environment, the expression of stress proteins subsides and allosuppression is restored by the sequestration of Sup35p into 'aggregates' by residual *[PSI⁺]* seeds.

growth under adverse conditions. Indeed, the reversible antisuppression induced by stress may serve to reduce termination codon readthrough, thereby preventing the harmful production of extended polypeptides. The *FLO8* gene has been identified as a likely 'regulated' template in the strain S288C (Liu *et al.*, 1996; Lindquist, 1997). This gene product is essential for filamentous growth, and yet this strain bears an apparently 'internal' termination codon. Whilst *[PSI⁺]*-induced allosuppression would be expected to elicit the expression of 'full-length' Flo8p and permit filamentous growth of S288C, we predict that the environmental conditions that normally induce filamentous growth would also invoke the transient shift of the *[PSI⁺]* strain to an antisuppressor state, thereby reducing nonsense suppression of termination codons and precluding pseudohyphae formation.

As proposed by Lindquist (1997), the interplay between stress proteins (Hsp104p) and prions does provide a plausible molecular mechanism for a cell to respond to its environment with a heritable change in phenotype. We would expand this concept to suggest that the yeast prion *[PSI⁺]* actually represents a mechanism for the regulation of translation termination efficiency in response to changes in enviromental conditions, rather than a simple switching between a *[PRION⁺]* and *[prion⁻]* heritable phenotype. Cells possess specific mechanisms for tolerating adverse environmental conditions and for the recovery and elimination of stress-denatured proteins. One such mechanism in *S.cerevisiae* involves the action of the stress protein Hsp104p, which is known to be important for protecting yeast against the detrimental effects of heat and alcohol (Sanchez and Lindquist, 1990; Sanchez *et al.*, 1992; Piper, 1995). Moreover, Hsp104p is a prerequisite for the maintenance and propagation of *[PSI⁺]* (Chernoff *et al.*, 1995). We propose that the stress-mediated regulation of translation termination efficiency in *[PSI⁺]* strains arises from the increased levels of stress proteins which mediate the partial regeneration of functional Sup35p and hence a transient increase in translation termination efficiency. Thermal and ethanol stress induce the elevated expression of Hsp104p and Ssa1p (Hsp70p) (Mager and Ferreira, 1993; Piper, 1995), which is known to result in the loss of the loss of *[PSI⁺]*-induced allosuppression (Chernoff *et al.*, 1995; Paushkin *et al.*, 1996). We postulate that Hsp104p is the primary factor in *[PSI⁺]*-meditated regulation of translation termination efficiency, in response to environmental stress. Clearly, not all stress-induced proteins have a role in this phenomenon, e.g. Hsp26p (this study). As a regulatory mechanism, the interplay between the stress proteins and the prion protein is such that the *[PSI⁺]* determinant is not eliminated from the cell. Upon removal of the environmental stress and the subsequent decrease in the intracellular level of stress proteins, Sup35p is sequestered by residual 'seeds' into large intracellular polymers. The corresponding depletion of soluble Sup35p results in a decrease in translation termination efficiency and the restoration of the *[PSI⁺]* allosuppressor phenotype (Figure 7).

Materials and methods

Strains

Three *[PSI⁺]* strains were selected on the basis of their difference in genetic background and for the presence of two specific genetic elements: (i) the ochre suppressor serine-inserting tRNA encoded by the *SUQ5* gene; and (ii) the *ade2-1* mutation, which permit a direct visualization of allosuppression status by colony colour (Cox, 1965). Strains exhibiting efficient translation termination give rise to red colonies, whereas those strains displaying an allosuppressor phenotype (i.e. termination inefficient) grow white. The three strains were rendered prion-deficient (i.e. *[psi⁻]*) on the basis of a stable white to red colony colour change that occurs upon growth on media containing 2.5 mM guanidine hydrochloride (Tuite *et al.*, 1981).

The genotypes of the four strains used in these studies were: BSC 783/4a, *SUQ5, ade2-1, ura3-1, his3-11, his3-15, leu2-3, leu2-112, MAT*a; BSC 783/4c, *SUQ5, ade2-1, ura3-1, his3-11, his3-15, leu2-3, leu2-112, MAT*a; BSC772/9d, *SUQ5, ade2-1, ura3-1, his4-166, leu2-2, lys1-1, can1-100, MAT*a; and MT 766/12a, *SUQ5, ade2-1, ura3-1, hsp26::HIS3, MAT*a

Growth media

Yeast strains were grown either in liquid YEPD complete medium [2% (w/v) glucose, 1% (w/v) Bacto-peptone, 1% (w/v) yeast extract] or defined minimal medium (0.67% Difco defined minimal medium without amino acids, 2% glucose) supplemented with the appropriate amino acids and cofactors (20 mg/l, except adenine which was added at 200 mg/l). For colony colour enhancement, yeast strains were grown on 1/4YEPD solid medium [4% (w/v) glucose, 1% (w/v) Bacto-peptone, 0.25% (w/v) yeast extract, 2% (w/v) agar]. The reduced content of yeast extract in 1/4YEPD ensures the maximum accumulation of the chromogenic adenine biosynthetic precursor. For studies using ethanol-supplemented media, strains were grown in flasks sealed with rubber bungs to reduce evaporation of the alcohol from the medium.

Sedimentation analysis of polypeptide release factors

Yeast strains were grown to an OD_{600} of 0.5, harvested and lysed with glass beads at 4°C, in buffer [25 mM Tris–HCl pH 8, 150 mM NaCl, 5 mM EDTA, 5 mM phenylmethylsulfonyl fluoride (PMSF), complete protease inhibitor mix (Boehringer Mannheim, one tablet per 5 ml of buffer)]. Lysates were then clarified by centrifugation for 10 min at 13 000 *g* and stored on ice. Samples of lysate and clarified lysate were boiled for 10 min, after the addition of 5× SDS–PAGE sample buffer [2.5 ml of glycerol, 1.25 ml of 20% (v/v) SDS, 0.25 ml of 2-mercaptoethanol, 1.25 ml of 0.5 M Tris–HCl pH 6.8]. Samples were cooled on ice and then spun for 5 min at 13 000 *g*. Protein samples were analysed using 10% SDS–polyacrylamide gels and electrophoretically transferred to nitrocellulose for Western blot analysis, employing polyclonal sera raised against yeast Sup35p and Sup45p expressed in *Escherichia coli*, as described previously (Stansfield *et al.*, 1995a).

Thermotolerance assays

Strains were grown at 25°C to mid-exponential phase in YEPD (OD_{600} 0.4) and then transferred to 37°C. Following a 1 h pre-treatment, strains were diluted to a density of ~3.5×10^3 cells/ml. Strains were then transferred to a 52°C shaking water bath, whereupon aliquots were removed at regular intervals and stored on ice. Aliquots of 150 µl (~500 cells) were plated in triplicate onto solid YEPD agar, and viable counts were determined after 5 days growth at 30°C.

Chemotolerance assays

A fresh colony of each strain was taken up from a 1/4YEPD plate, resuspended in 500 µl of sterile water and diluted to ~1×10^6 cells/ml. Then, 8×2 µl strain suspensions were spotted onto a chemical gradient plate. Each plate comprised 50 ml of 1/4YEPD agar supplemented with a stress reagent, which was poured 24 h prior to use. The stress agar was allowed to set with the plate tilted, so that there was no stress agar at one end of the plate. The plates were used following the addition of 50 ml of 'top' 1/4YEPD agar as soon as the top agar had set. Following the spotting of the strain suspensions, all gradient plates were sealed to prevent evaporation of stress-inducing agents and then incubated for 5 days at 30°C.

Quantifying allosuppression levels in vivo

Yeast strains were transformed with one of the two 'readthrough' vectors pUKC815 or pUKC817 (Stansfield *et al.*, 1995b). Essentially, the single-copy control vector pUKC815 carries the *lacZ* gene under the control of the constitutive *PGK* promotor. The readthrough vector pUKC817 has an in-frame premature ochre (TAA) termination signal. Strains were grown in appropriate minimal media to an OD_{600} of 0.45, whereupon the cells from 10 ml culture aliquots were harvested and stored at –20°C. Strains were grown in the presence of 7% (v/v) ethanol for at least 24 h, to ensure a true reflection of readthrough levels (i.e. stress exposure was prolonged to permit the 'turnover' of any residual β-galactosidase produced during growth in normal media). The degree of allosuppression was determined as a function of the β-galactosidase levels of the strains bearing the readthrough vectors. The cell pellets from the culture aliquots were resuspended in 600 µl of buffer Z (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, pH 7.0) supplemented with EDTA (5 mM final), 2-mercaptoethanol (50 mM final) and PMSF (5 mM final). Cells were lysed at 4°C by vortexing three times for 30 s (with 30 s intervals on ice) in the presence of an equal volume of glass beads. β-Galactosidase and Bradford assays were then performed on lysates clarified by centrifugation (10 min, 13 000 *g*, 4°C) as described previously (Stansfield *et al.*, 1995b).

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