Yeast Prion [Ψ+] and Its Determinant, Sup35p

By Tricia R. Serio, Anil G. Cashikar, Jahan J. Moslehi, Anthony S. Kowal, and Susan L. Lindquist

Introduction

[Ψ+] and [URE3] are two non-Mendelian genetic elements of the yeast Saccharomyces cerevisiae that appear to be inherited through an unusual mechanism: the continued propagation of an alternate protein conformation. The protein determinants of these elements, Sup35p for [Ψ+]\(^1\)\(^2\) and Ure2p for [URE3]\(^3\)\(^4\) have the unique ability to exist in at least two different, stable conformations in vivo.\(^4\)\(^5\) Although the spontaneous generation of one conformer is rare, this alternate form, once acquired, becomes predominant, influencing the other conformer to change states.\(^5\) This self-perpetuation of protein conformation is the key to the non-Mendelian inheritance of both [Ψ+] and [URE3]. In addition, the [Het-S] phenotype of Podospora anserina, another fungus, may be inherited by a similar mechanism.\(^9\) This article focuses on both in vivo and in vitro methods used to analyze [Ψ+], the most extensively studied member of this group.

Genetics of [Ψ+] Inheritance

[Ψ+] was originally described in 1965 by Cox as a translation infidelity factor.\(^10\) Translation terminated efficiently at nonsense codons in strains classified as [psi−], whereas [Ψ+] strains were capable of omnipotently

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suppressing nonsense mutations by increasing the rate at which ribosomes read through stop codons. The \([PSI^+]\) phenotype, translational read through, has been monitored most frequently in yeast strains harboring nonsense mutations in metabolic enzymes by growth on defined medium lacking the product of that pathway. In these cases, \([psi^-]\) yeast strains are auxotrophic for that nutrient and will not grow in the absence of supplements, whereas \([PSI^+]\) yeast strains are at least partially prototrophic for the nutrient.

This convenient method of screening for \([PSI^+]\) formed the basis of early genetic characterizations, which revealed the unique properties of \([PSI^+]\) inheritance. Crosses between haploid \([psi^-]\) and \([PSI^+]\) strains yield \([PSI^+]\) diploids, indicating that the \([PSI^+]\) state is dominant in vivo (Fig. 1A). Surprisingly, the meiotic haploid progeny of these \([PSI^+]\) diploids are all \([PSI^+]\), demonstrating that \([PSI^+]\) was propagated by a non-Mendelian mode of inheritance (Fig. 1A). This idea is supported further

A. Mating

\[
\begin{array}{cc}
\psi^+ & \psi^- \\
N & \text{X} & \text{X} \\
2N & \psi^+ \\
N & \psi^+ & \psi^+ & \psi^+ & \psi^+ \\
\end{array}
\]

B. Cytoduction

\[
\begin{array}{cc}
\psi^+ & \psi^- \\
N & \text{X} & \text{X} \\
2N & \psi^+ \\
N & \psi^+ & \psi^+ & \psi^+ & \psi^+ \\
\end{array}
\]

Fig. 1. Genetic analysis of \([PSI^+]\) inheritance. (A) Shown are yeast cells that are \([PSI^+]\) (\(\psi^+\), gray) or \([psi^-]\) (\(\psi^-\), white) with hatched nuclei. Mating (X) produces a \([PSI^+]\) diploid (\(\psi^+, \text{gray}\)), and subsequent sporulation (four arrows) yields four haploid segregants that are all \([PSI^+]\). (B) \([PSI^+]\) and \([psi^-]\) yeast cells as in (A). Cytoduction allows cytoplasmic mixing in the absence of nuclear fusion (note separate hatched nuclei). Segregants are all \([PSI^+]\) (\(\psi^+, \text{gray}\)). Ploidy for both (A) and (B) is shown to the left of the figure (\(N = \text{haploid}, 2N = \text{diploid}\)).
by the observation that [$PSI^+$] could be transmitted to susceptible strains by cytoduction experiments in which cytoplasmic mixing occurs in the absence of nuclear fusion (Fig. 1B).\textsuperscript{11}

Efforts to link [$PSI^+$] to extrachromosomal plasmids or cytoplasmically propagated nucleic acids proved fruitless\textsuperscript{12,13} and were complicated further by another puzzling aspect of [$PSI^+$] inheritance. [$PSI^+$] is a metastable genetic element; [$PSI^+$] is lost at a strain-specific characteristic low rate (see later) through mitotic division, but it can also reappear spontaneously in these [$psi-$] strains.\textsuperscript{14} This “reversible curing” is inconsistent with a nucleic acid-directed inheritance model. Furthermore, treatments that are nonmutagenic to nucleic acids, such as growth in the presence of 5 mM guanidine hydrochloride are efficient at curing [$PSI^+$].\textsuperscript{14}

The nature of the [$PSI^+$] element remained a mystery for nearly 30 years until Wickner suggested that [$PSI^+$] and [$URE3$] were propagated by alternate protein conformations rather than nucleic acids.\textsuperscript{3} Soon after this idea was proposed, a link between [$PSI^+$] propagation and the molecular chaperone heat shock protein 104 (Hsp104) was established. Either deletion or transient overexpression of Hsp104 is sufficient to convert yeast strains from [$PSI^+$] to [$psi-$].\textsuperscript{5,15} That the transient overexpression of a molecular chaperone, whose only known function is to alter the physical state of substrate proteins, could induce a heritable change in phenotype in yeast provides one of the strongest arguments to date in support of a protein-only mode of inheritance for [$PSI^+$].

Sup35p, Protein Determinant of [$PSI^+$]

Identification of the yeast protein Sup35p as the determinant of [$PSI^+$] provided the first step in understanding the basis of the [$PSI^+$] phenotype and its propagation. Sup35p is the yeast homolog of the eukaryotic release factor eRF3.\textsuperscript{16–18} Sup35p forms a functional translation termination complex

\textsuperscript{11} B. S. Cox, M. F. Tuite, and C. S. McLaughlin, Yeast 4, 159 (1988).
\textsuperscript{13} C. S. Young and B. S. Cox, Heredity 28, 189 (1972).
\textsuperscript{14} M. F. Tuite, C. R. Mundy, and B. S. Cox, Genetics 98, 691 (1981).
with the yeast eRF1 homolog, Sup45p. Together, Sup35p and Sup45p
direct the faithful termination of translation at stop codons in [\(psi^-\)]
cells. In [\(PSI^+\)] cells, however, the Sup35p function is compromised, leading
to the nonsense suppression phenotype. This epigenetic loss of Sup35p
activity may result from a unique type of Sup35p aggregation. These
aggregates have only been isolated from [\(PSI^+\)] strains and are lost by
treatments that cure [\(PSI^+\)], such as the deletion or overexpression of Hsp104.

Work by several groups has been instrumental in elucidating the regions
of Sup35p important for translation termination and [\(PSI^+\)]
propagation. Sup35p is composed of three regions—N, M, and C (Fig. 2A)—based on
amino-terminal composition and homology to other translation factors. The
amino-terminal region, N [amino acids (aa) 1–123], has an unusual amino
acid composition and distribution, with 78% of all residues being glycine
(G), tyrosine (Y), asparagine (N), or glutamine (Q). This region contains six
imperfect repeats of the sequence QGGYQ(Q)QYNP. N is the prion-
determining domain of Sup35p: it is necessary for the propagation of
[\(PSI^+\)], and transient overexpression of this region alone is sufficient
to induce new [\(PSI^-\)] elements in all [\(psi^-\)] strains expressing full-length
Sup35p. N has a high propensity for self-association: when expressed
as an isolated domain in yeast, it is always aggregated. In addition, N has
been shown to be highly amyloidogenic in vitro. It is insoluble in physiologi-
cal buffers and forms amyloid even in the presence of denaturant. The
glutamine-rich repeats present in N play a central role in the self-assembly
of N both in vivo and in vitro.

The M region of Sup35p (aa 124–253) is highly charged. Notably, the
charged residues are strongly biased to two amino acids: glutamic acid
(18%) and lysine (19%), with no arginines present and aspartic acid
comprising only a minor fraction (5%). Although not essential for the induction
of [\(PSI^+\)], M appears to enhance the solubility of the prion-determining
N region, profoundly altering its behavior both in vivo and in vitro.

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19 V. V. Kushnirov, M. D. Ter-Avanesyan, M. V. Telckov, A. P. Surguchov, V. N. Smirnov,
20 Y. O. Chernoff, M. V. Ptyushkina, M. G. Samsonova, G. I. Sizonencko, Y. I. Pavlov,
A. Sup35p

**Amino Acid Composition**

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<td>Y</td>
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<td>N</td>
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<td>Q</td>
<td>29%</td>
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<tr>
<td>E</td>
<td>18%</td>
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<tr>
<td>K</td>
<td>19%</td>
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**Properties**

- dispensable for viability
- minimal requirement for Ψ+ induction in vivo
- minimal requirement for amyloid formation in vitro
- profoundly influences the ability of N to induce Ψ+ in vivo and to remain soluble in aqueous buffers in vitro
- essential for viability
- homology to EF-1α
- functions in translation termination as eRF3
- putative GTPase

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B. Expression Constructs

**E. coli expression**

Solubility in E. coli

- Fiber Formation

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<th>Solubility</th>
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**Yeast expression**

Ψ+ Induction

- +
- -

Fig. 2. (A) Biological and physical characteristics of Sup35p. The schematic diagram of Sup35p depicts amino-terminal (N), middle (M), and carboxy-terminal regions. The amino acid boundaries of these regions, as well as their unusual amino acid compositions, are indicated to the left of the diagram (G, glycine; Y, tyrosine; N, asparagine; Q, glutamine; E, glutamic acid; and K, lysine). Horizontal lines indicate the nonapeptide repeats of N, the plus and minus symbols reflect the charged character of M, and GTP indicates consensus GTP-binding sites in C. A brief summary of the roles of these regions in [PSI+] metabolism and translational termination is indicated to the right of the diagram. (B) Constructs for the expression of Sup35p and subfragments in E. coli and yeast. Schematic diagrams of expression constructs for N, M, C, or NM in E. coli are shown in the top half along with their solubilities in E. coli lysates and their ability to form amyloid fibers in vitro. Black rectangles indicate the position of a 10-residue His tag. Schematic diagrams of constructs used for the expression of NM<s<GFP, NMCsGFP, or sGFP from an extrachromosomal plasmid in yeast are indicated in the bottom half. The abilities of these constructs to induce Ψ+ in the presence of full-length, wild-type Sup35p expressed from the genome are indicated to the right. A schematic of full-length Sup35p, including amino acid residue numbers, is shown at the top for reference.
In contrast to N, NM can exist in multiple states \textit{in vivo}, modeling the differences in Sup35p solubility that are characteristic of \([PSI^+]\) and \([psi^-]\) strains.\(^{5,6,27}\) In addition, purified NM forms amyloid slowly in physiologic buffers.\(^{22}\) NM assembly is accelerated by the addition of preformed NM amyloid or lysates from \([PSI^+]\) but not \([psi^-]\) cells,\(^{22}\) linking the properties of amyloid formation \textit{in vitro} to the propagation of the \([PSI^+]\) state \textit{in vivo}. This ability of NM in one conformation to influence the physical state of the same protein in another conformation is the basis of protein-conformation self-perpetuation and the protein-only mode of inheritance for \([PSI^+]\).

The carboxy-terminal region of Sup35p, C (aa 254–686), has sequence homology to the yeast translation elongation factor, EF-1\(\alpha\).\(^{19}\) This region complexes with Sup45p,\(^{17,18}\) contains several putative GTP-binding sites,\(^{19}\) and functions in translational termination.\(^{17,18,28}\) Unlike N and M, this region is essential for viability.\(^{29}\) The epigenetic modulation of Sup35p carboxy terminus activity in translation termination is the \([PSI^+]\) phenotype. Consequently, the carboxy terminus of Sup35p, linked to N and M, must be expressed in all \([PSI^+]\) strains.

Analysis of \([PSI^+]\) \textit{in Vivo}

A guide to general yeast genetic techniques may be found elsewhere in this series.\(^{30}\) This section discusses variations on those techniques that are particular to the study of \([PSI^+]\) \textit{in vivo}.

Reversibly curable nonsense suppression, exhibiting non-Mendelian, cytoplasmic inheritance, is the most commonly used test for \([PSI^+]\). Suppression of nonsense mutations in auxotrophic markers, such as \(ade2-1\) or \(adel-14\), resulting in growth on defined medium lacking adenine (SD-Ade), has been the most convenient and well-accepted assay for \([PSI^+]\). Although the suppression of nonsense mutations in any metabolic enzyme and selection in the same manner are equally useful, analysis of strains harboring nonsense mutations in the adenine biosynthetic pathway provides an additional color selection assay. If grown on complete medium (YPD), \([psi^-]\) strains carrying a nonsense mutation in the adenine pathway form red

colonies, whereas isogenic \([\Psi^+]\) strains form white or pink colonies (see later). This color readout of \([\Psi^+]\) is diminished if the complete medium is supplemented with extra adenine or the antibiotic tetracycline\(^2\); therefore, growth on minimal media (SD-Ade) should always be assessed in parallel.

The growth assay, as well as the color assay, for \([\Psi^+]\) described earlier is influenced by the "strength" of suppression, the efficiency with which nonsense codons are read through, and the mitotic stability of the phenotype. In most cases, growth of a \([\Psi^+]\) strain on SD-Ade, for example, will not be observed for 7–10 days at 25°C, whereas a wild-type yeast strain, prototrophic for adenine, will grow within 2 days. For some markers, the efficiency of read through may not produce enough product to support growth in the absence of supplements. The strength of suppression can be increased, in some cases, by growth at lower temperatures (25°C versus 30°C) or on alternate carbon sources (i.e., ethanol). These characteristics are yeast strain specific but should be considered when monitoring \([\Psi^+]\).

In addition, others have described a variation in \([\Psi^+]\) suppression within a single genetic background.\(^2\) For example, when a single \([\Psi^+]\) yeast strain containing a \([\Psi^+]\)-suppressible nonsense mutation in the \(ADE1\) gene is plated on YPD, most colonies are white; however, a few colonies that are different shades of pink are also observed. These isolates are characterized by different strengths of suppression and different mitotic stabilities, with white being the strongest in both cases. If one colony of any color is picked and replated, most colonies will maintain that same color, but colonies of different colors are also observed as before. The continued ability of different isolates to produce colonies with a spectrum of suppression strengths suggests that the variation between isolates is non-Mendelian in nature. For this reason, \([\Psi^+]\) variants isolated from a single genetic background are referred to as "strains" of \([\Psi^+]\).\(^2\) \([\Psi^+]\) strains, which are isogenic, should not be confused with yeast strains that are genetically distinct.

Monitoring read through of nonsense mutations may also be complicated by differences in yeast strain backgrounds. For example, not all nonsense codons will be suppressed in all \([\Psi^+]\) yeast strains. Although the context of the nonsense mutation certainly plays a role in the efficiency of suppression,\(^3\)\(^4\) additional trans-acting factors, known as allosuppressors, have also been implicated.\(^3\)\(^5\) For example, \([\Psi^+]\) was originally described

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as a factor capable of suppressing the ade2-1 (UAA) allele in the presence of the allosuppressor SUQ5, a serine-inserting, UAA-specific tRNA.\textsuperscript{33} Allosuppressors have been isolated in some but not all [\textit{PSI}^+\textit{\textsuperscript{\textdagger}}] strains, but interactions with \textit{trans} regulators should be keep in mind when initially characterizing new strains for [\textit{PSI}^+\textit{\textdagger}] status. For this reason, transmission to a [\textit{PSI}^+\textit{\textdagger}]-susceptible strain by cytoduction is perhaps the most reliable test.

Although commonly used, the nonsense suppression assay for [\textit{PSI}^+\textit{\textdagger}] is complicated by the need to support growth. To circumvent this problem, a quantitative nonsense suppression assay has also been developed that can be employed in any strain regardless of the auxotrophic markers available.\textsuperscript{36} This assay is dependent on expression of a translational fusion between phosphoglycerate kinase and \(\beta\)-galactosidase. The two open reading frames are expressed in a single transcriptional unit but are separated by one of the three nonsense codons. Suppression of the nonsense mutation leads to the production of \(\beta\)-galactosidase, and the level of suppression may be quantitated by activity of this enzyme.

When working with [\textit{PSI}^+\textit{\textdagger}], it is of the utmost importance to continually reconfirm the [\textit{PSI}^+\textit{\textdagger}] status of yeast strains by a combination of experimental tests in addition to nonsense suppression. Suppression should be curable by treatment with guanidine hydrochloride or by the deletion and over-expression of Hsp104.\textsuperscript{5,14,15} In addition, suppression should be dominant in diploids\textsuperscript{10} and transmissible by cytoduction to susceptible yeast strains.\textsuperscript{11} These additional analyses will avoid isolation of revertants of the nonsense mutation being monitored in a growth or color assay as well as mutations in other factors important for translational fidelity.

Characterization of mutations within the Sup35 coding sequence has been used to increase our understanding of the \textit{cis}-acting requirements for [\textit{PSI}^+\textit{\textdagger}] induction and propagation in the presence of a wild-type copy of Sup35.\textsuperscript{1,24,25,37} Although additional work of this type will continue to increase our knowledge, several potential pitfalls are important to avoid. For example, work has indicated that while some mutations in Sup35p are capable of forming aggregates \textit{in vivo} and/or amyloid \textit{in vitro}, they do not induce new [\textit{PSI}^+\textit{\textdagger}] elements or support [\textit{PSI}^+\textit{\textdagger}] propagation in the presence of wild-type Sup35p \textit{in vivo}.\textsuperscript{24,25,38} These experiments indicate that compatibility between endogenous and exogenous Sup35p, as well as the efficiency of protein incorporation into aggregates, is critical for suppression and heritability. Consequently, both of these factors have the potential to complicate the analysis of Sup35p mutants. Replacement of endogenous Sup35p with

\textsuperscript{38} N. V. Kochneva-Pervakhova, \textit{EMBO J.} 17, 5805 (1998).
generated mutants may aid in their analysis. Because Sup35 is an essential gene, replacement of mutant alleles with wild-type sequences provides additional insight into the functional state of the molecule.

Finally, analysis of [PSI⁺] induction is complicated by a non-Mendelian factor, [PIN⁺]. [PIN⁺] stands for [PSI⁺] inducible. [PSI⁺] can be induced in [PIN⁺] [psi⁻] yeast strains by the overexpression of any fragment of Sup35p containing N, whereas yeast strains that are [pin⁻][psi⁻] can become [PSI⁺] only by overexpression of the N region of Sup35p alone. Curing of [PSI⁺] by growth on guanidine hydrochloride produces both [pin⁻] and [PIN⁺] isolates, whereas curing by the overexpression of Hsp104 seems to only produce [PIN⁺] strains. In addition, the [PIN⁺] status may spontaneously change to [pin⁻], especially if strains are kept at 4° for extended periods of time. Care should therefore be taken to ensure that strains are susceptible to [PSI⁺] induction with wild-type copies of Sup35p in parallel with any unknowns.

Analysis of Sup35p in Yeast Cells

The first biochemical evidence that the [PSI⁺] phenotype was propagated by a protein-only mode of inheritance was provided by analysis of the physical state of Sup35p in [psi⁻] and [PSI⁺] cells. Sup35p is largely soluble in [psi⁻] cells but is mostly insoluble in [PSI⁺] cells. Conversion between these physical states, invoked by transient changes in either the concentration of Sup35p or the molecular chaperone Hsp104, results in a heritable change in phenotype between [PSI⁺] and [psi⁻] or vice versa. Two types of analyses, one in living yeast cells and one in yeast extracts, have been seminal in providing support for [PSI⁺] as a yeast prion.

Analysis of Sup35p Tagged with Green Fluorescent Protein in Living Yeast Cells

Translational fusions of full-length Sup35p or NM to the green fluorescent protein (GFP) or GFP containing amino acid substitutions (S65T, V164A) that increase fluorescence and decrease self-association (superglow

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39 I. Derkatch and S. Liebman, unpublished observation.
GFP, sGFP\textsuperscript{42,43} have provided an accurate model system for monitoring the aggregation state of full-length Sup35p in living cells.\textsuperscript{5} Following short induction times for expression of these fusion proteins, fluorescence is diffuse in \([psi^-]\) strains and coalesces rapidly in \([PSI^+]\) strains, whereas fluorescence from GFP or sGFP alone remains diffuse in both \([psi^-]\) and \([PSI^+]\) cells (Fig. 3A). We typically observed a single focus with GFP fusions, but multiple foci are visible with sGFP, most likely due to the enhanced fluorescence and decreased propensity for self-aggregation of sGFP. This system accurately mimics all aspects of \([PSI^+]\) metabolism.\textsuperscript{5} Prolonged overexpression of NMGFP (or NMsGFP) or Sup35GFP (or Sup35sGFP) in \([psi^-]\) strains will ultimately lead to the coalescence of Sup35p and the induction of new \([PSI^+]\) elements,\textsuperscript{5} but in the time courses described next, an unambiguous difference in fluorescence between \([psi^-]\) and \([PSI^+]\) strains is observed.

Construction of Expression Plasmids. We have constructed low- and high-copy number plasmids for either the constitutive or the inducible expression of Sup35p and NM fused to GFP or sGFP,\textsuperscript{5,27} but we will limit the discussion here to the copper-inducible low-copy number vectors expressing translational fusions to sGFP. The parent plasmids for these constructs are the ampicillin-resistant, \(URA3^+\), CEN plasmid: pRS316.\textsuperscript{44} The copper-inducible CUP1 promoter\textsuperscript{45} was cloned between the \textit{EcoRI} and the \textit{BamHI} sites of this plasmid by polymerase chain reaction (PCR) to generate 316CUP1. A cassette for expression of sGFP from the CUP1 promoter was also generated by PCR and cloned between the \textit{SacI} and the \textit{SacII} sites of 316CUP1 to yield 316CG. Finally, full-length Sup35p, lacking its natural stop codon, or NM was amplified by PCR and cloned between the \textit{BamHI} and the \textit{SacI} sites of 316CG to yield the expression vectors Sup35GFP (Sup35sGFP) or CNMG (NMsGFP), respectively.

Induction and Analysis of Sup35p Fusions to sGFP. Plasmids are transformed into isogenic \([PSI^+]\) and \([psi^-]\) yeast strains by the lithium acetate method, for example, and selected for on SD medium lacking uracil. Under certain circumstances, low-level expression from the CUP1 promoter was also generated by PCR and cloned between the \textit{SacI} and the \textit{SacII} sites of 316CUP1 to yield 316CG. Finally, full-length Sup35p, lacking its natural stop codon, or NM was amplified by PCR and cloned between the \textit{BamHI} and the \textit{SacI} sites of 316CG to yield the expression vectors Sup35GFP (Sup35sGFP) or CNMG (NMsGFP), respectively.

\textsuperscript{44} R. S. Sikorski and P. Hieter, \textit{Genetics} \textbf{122}, 19 (1989).
A

\[
\begin{array}{c|c|c|c}
N & M & C & sGFP \\
\hline
\end{array}
\]

\[\psi^-\quad \psi^+\]

\[
\begin{array}{c|c|c|c}
N & M & sGFP \\
\hline
\end{array}
\]

\[\text{sGFP}\]

B

\[
\begin{array}{c|c|c|c}
\psi^- & \psi^+ & \psi^- & \psi^+ \\
\hline
\text{Sup35} & & & \\
\text{Ribosomal protein L3} & & & \\
\text{Hsp70, Hsc70} & & & \\
\text{S} & \text{P} & \text{S} & \text{P} \\
\hline
\end{array}
\]

\[12,000g\]

FIG. 3. (A) Monitoring aggregation in living yeast cells. Fluorescence from sGFP or NMC (NMCsGFP) and NM (NMsGFP) tagged with sGFP was monitored in 74-D694 \(\Psi^-\) and \(\Psi^+\) strains 4 hr after induction with copper sulfate. At this point, coalesced fluorescence is observed only in the \(\Psi^+\) strain from NMCsGFP or NMsGFP. (B) Solubility of Sup35p in yeast lysates. Immunoblots of yeast lysates from 74-D694 \(\psi^-\) or \(\psi^+\) strains following differential centrifugation at 12,000g are shown, Polyclonal rabbit antisera to Sup35p or ribosomal protein L3 or a rat monoclonal antibody to Hsp70, Hsc70 (MAb 7.10) were used as probes. S, supernatant fraction; P, pellet.
are below the level of detection for fluorescence microscopy. Single colonies are inoculated into a liquid culture in SD-Ura at 30\(^\circ\) with constant agitation on a roller drum (60 rpm) to a density of \(\sim 1 \times 10^6\) cells/ml. Copper sulfate (CuSO\(_4\)) is added to a final concentration of 50 \(\mu M\) to induce expression from the CUP1 promoter under the same growth conditions. Cells are viewed under blue light at 100\(\times\) magnification 4 hr after induction.

**Differential Centrifugation Analysis of Sup35p in Yeast Extracts**

Another method to analyze the physical state of Sup35p in yeast is differential centrifugation. In the most simple case, Sup35p from \([PSI^+]\) lysates partitions to the pellet fraction whereas Sup35p from \([psi^-]\) lysates is found predominantly in the soluble fraction (Fig. 3B).\(^5\) Unlike the behavior of Sup35p, the location of other proteins, such as the ribosomal protein L3 or Hsp70 and Hsc70, is not altered in a \([PSI^+]-\)dependent manner. Because of the great difficulty in working with aggregation-prone proteins, considerable time should be invested to ensure reproducibility with known samples before analyzing the behavior of unknowns. The method described here involves centrifugation of the lysate alone,\(^5\) whereas methods described by others have utilized sucrose cushions and gradients with similar results.\(^6\) As with all fractionations, introduction of either cushions or gradients diminishes cross-contamination between fractions. However, the high-speed centrifugation step included in the sucrose cushion method to remove unbroken cells has the disadvantage of removing some fraction of Sup35p aggregates from \([PSI^+]\) lysates. The degree of purity and yield required for subsequent analysis should, therefore, dictate the method employed. All of these methods are also suitable for the analysis of Sup35p fragments expressed in yeast.\(^5,6,27\)

**Culture Growth and Extract Preparation**

1. Grow yeast cultures to midlog phase [\(\sim 1-5 \times 10^7\) cells/ml in complete (YPD) or \(\sim 2-4 \times 10^6\) cells/ml in synthetic (SD) medium] at 30\(^\circ\) with constant shaking (250 rpm). This procedure, however, has yielded the same results with stationary-phase cultures. Fifteen minutes prior to collection, add cyclohexamide to 200 \(\mu g/ml\) to stabilize polysomes and allow newly synthesized proteins to achieve their characteristic conformations.

2. Cool the cells on ice for 15 min and then harvest by low-speed centrifugation (2000g, 5 min, 4\(^\circ\)). Discard the supernatant and wash the cell pellet once with an equal volume of cold water containing 200 \(\mu g/ml\) cyclohexamide.
3. Wash the pellet once with an equal volume of lysis buffer [50 mM Tris–HCl (pH 7.5), 5 mM MgCl₂, 10 mM KCl, 0.1 mM EDTA (pH 8.0), 1 mM dithiothreitol (DTT), 100 μg/ml cyclohexamide, 1 mM benzamidine, 2 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml leupeptin, 2 μg/ml pepstatin A, 100 μg/ml ribonuclease A].

4. Transfer cells to a 1.5-ml microcentrifuge tube and pellet at (2000g, 5 min, 4°C). If desired, the cells may be flash frozen and stored at −80°C at this point.

5. Resuspend pellet in lysis buffer at a concentration of ~3 × 10⁶ cells/μl. Add an equal volume of 425- to 600-μm acid-washed glass beads (Sigma, St. Louis, MO).

6. Homogenize cells in a Mini Bead Beater 8 (Biospect Products) for approximately 4 min at 4°C. Monitor cell breakage by light microscopy.

7. Puncture the bottom of the tube with a 18-gauge needle and place a smaller tube into a 12 × 75-mm round-bottom polypropylene tube (Falcon: Becton Dickinson, Franklin Lakes, NJ). Centrifuge at 100g for 1 min at 4°C to separate lysate from glass beads.

8. Wash glass beads twice with 1/2 volume of lysis buffer originally used. Combine washes with lysate from step 7.

9. Preclear the lysate at 2,500g for 10 min at 4°C to pellet unbroken cells. Remove the supernatant to a new tube without disturbing the pellet.

10. Determine the protein concentration of the lysate. Typical yields are 5–15 mg/ml using Bio-Rad (Richmond, CA) protein assay reagent with bovine serum albumin (BSA) as a standard. Lysates may be flash frozen and stored at −80°C at this point.

**Differential Centrifugation Analysis**

1. Separate aggregates from soluble protein in a fraction of the lysate by centrifugation at 6000–12,000g for 10 min at 4°C.

2. Remove supernatant to a tube containing an appropriate volume of 6× sample buffer [350 mM Tris–HCl (pH 6.8), 30% (v/v) glycerol, 10% (w/v) SDS, 600 mM DTT, 0.12% (w/v) bromphenol blue] to give a 1× concentration. This is the supernatant fraction.

3. Resuspend the pellet in the same volume of lysis buffer used in step 1 of this section. Transfer resuspended pellet to a new tube containing an appropriate volume of 6× sample buffer to give a 1× concentration. This is the pellet fraction.

4. Incubate supernatant and pellet samples as well as a total lysate sample at 100°C in a water bath for 10 min.
5. Separate proteins on a 10% SDS-PAGE (25 mA/gel), electrotransfer to Immobilon-P (Millipore, Bedford, MA), and analyze by immuno blotting. A total of 36 μg of protein/lane yields a sufficient Sup35p signal for detection with our antiserum.\(^5\) In addition, we typically analyze fractionation of ribosomes by immunoblotting with an antibody to the ribosomal protein L3.\(^5\) Detection with either \(^{125}\)I-labeled protein A (Amersham, Arlington Heights, IL) followed by autoradiography or protein A–peroxidase (Boehringer-Mannheim, Indianapolis, IN) followed by ECL (enhanced chemiluminescence, Amersham) yield, similar results.

Analysis of Sup35p Purified from *Escherichia coli*

Recent work has linked the process of amyloid formation *in vitro* to the propagation of [\(\text{PSI}^+\)] *in vivo*. Fragments of Sup35p capable of inducing [\(\text{PSI}^+\)] *in vivo* form amyloid *in vitro*.\(^{22,23}\) Lysates from [\(\text{PSI}^+\)] but not [\(\text{psi}^-\)] strains accelerate the formation of amyloid *in vitro*, as do preformed fibers.\(^{22,23}\) Deletions within the prion-determining N region,\(^{22,24}\) as well as specific point mutations,\(^{24}\) slow the process of assembly into amyloid\(^{22,24}\) as well as block the induction of new [\(\text{PSI}^+\)] elements.\(^{24}\) Similarly, the expansion of repeated sequences in the N region accelerates amyloid formation *in vitro* and increases the efficiency of [\(\text{PSI}^+\)] induction *in vivo*.\(^{25}\)

We have assessed the abilities of N, M, NM and NMC, expressed and purified from *E. coli*, to form amyloid *in vitro* (Fig. 1B). Although fragments containing the N region are capable of this ordered assembly, we will only discuss the purification and analysis of the assembly process here for NM and NMC, as these fragments most accurately reflect [\(\text{PSI}^+\)] metabolism *in vivo*.\(^{5,26}\) Because NM and NMC form amyloid under native conditions,\(^{22}\) we routinely purify these fragments under denaturing conditions (8 M urea) to maintain the protein in a uniform state that is more amenable to studying the assembly process. The importance of obtaining a denatured, uniform solution of protein prior to initiating a kinetic analysis of amyloid formation cannot be stressed enough. Others have reported that protein purified in 6 M urea must be cleared of amyloid by filtration to obtain reproducible results.\(^{24}\) Procedures for purification under denaturing conditions in 8 M urea will be discussed here.

Expression Constructs

The expression of all cloned fragments of Sup35p is driven by T7 polymerase. Fragments were cloned into either pJC45 encoding an amino-terminal 10 residue histidine tag (His\(_{10}\)) or pJC25, lacking a tag.\(^{47}\) These

plasmids are high copy number, containing the pUC origin of replication, and have a consensus T7 promoter and the lacI operator at the 5' end of the multiple cloning site. Sup35p fragments were inserted between the NdeI and the BamHI sites, allowing in-frame fusion to His10 in the case of pJC45. The plasmids impart ampicillin resistance.

**Bacterial Growth and Induction**

Each construct is expressed in BL21 [DE3] pAP lacI<sup>q</sup>. This strain contains a [DE3] lysogen for the high-level expression of T7 polymerase following induction with isopropyl-\(\beta\)-D-thiogalactopyranoside (IPTG; Sigma). The strain also expresses a low level of the lacI product to repress leaky expression of the polymerase and, therefore, the target protein. The strain is kanamycin resistant.

Competent *E. coli* (BL21 [DE3] pAP lacI<sup>q</sup>) are transformed with expression plasmids by electroporation and selected on LB plates containing 50 \(\mu\)g/ml kanamycin and 200 \(\mu\)g/ml ampicillin. Fresh transformants should always be used for the expression of full-length Sup35p, as prolonged passage of the expression plasmid in this strain leads to a high degree of proteolysis.\(^{27}\) Proteolysis is not observed with NM, however, and glycerol stocks of the expression strain may be stored for months at \(-80^\circ\).\(^{27}\)

A single colony of BL21 [DE3] pAP lacI<sup>q</sup> containing the expression construct is inoculated into 1 liter of Circle Grow medium (Bio 101, Vista, CA) and incubated at 37 ° with constant shaking (300 rpm) until an \(\text{OD}_{600}\) of 0.8 is reached (approximately 7 hr). IPTG is added to 1 mM, and the culture is incubated further under the same conditions for 2 hr. Bacteria are then collected by centrifugation (3000g, 10 min, 4°). The pellet may be stored at \(-80^\circ\) or processed immediately. Again, storage of the pellet should be minimal for the purification of full-length Sup35p.

**Bacterial Lysis**

Cell pellets are lysed in 50 ml lysis buffer H [20 mM Tris–HCl (pH 8.0), 8 M urea] for each liter of culture for all His<sub>10</sub> proteins or in lysis buffer N [10 mM Tris–HCl (pH 7.2), 8 M urea] for nontagged NM. High-grade urea (Boehringer Mannheim) is prepared freshly for each purification to minimize covalent modifications to the protein due to the production of cyanate ions.\(^{48}\) The resuspended pellet is incubated for 30 min at 25° with occasional agitation. The lysate is then precleared by centrifugation at 30,000g for 20 min at 10°.

**Purification of His-Tagged NMC or NM**

**Buffers**

Lysis buffer H: 20 mM Tris–HCl (pH 8.0), 8 M urea  
Ni wash buffer: 20 mM Tris–HCl (pH 8.0), 8 M urea, 40 mM imidazole  
Ni elution buffer: 20 mM Tris–HCl (pH 8.0), 8 M urea, 400 mM imidazole  
Q wash buffer H: 20 mM Tris–HCl (pH 8.0), 8 M urea, 100 mM NaCl  
Q elution buffer H: 20 mM Tris–HCl (pH 8.0), 8 M urea, 300 mM NaCl

**Procedure.** All steps are carried out at 25°.

Precleared supernatants from cell lysates are applied to a 50-ml Ni²⁺-nitrilotriacetic acid agarose column (Ni²⁺-Nta; Qiagen, Valencia, CA) pre-equilibrated with lysis buffer H at a flow rate of approximately 3 ml/min. The column is washed with 5 bed volumes of Ni wash buffer, and the protein is eluted in a single step with 100 ml of Ni elution buffer. The eluate is applied directly onto a 20-ml Q Sepharose Fast Flow column (Pharmacia, Piscataway, NJ) pre-equilibrated with Ni elution buffer. The column is washed with 5 bed volumes of Q wash buffer H, and the protein is eluted in a single step with 45 ml of Q elution buffer H. The purification and purity of the final product are analyzed by 10% SDS–PAGE followed by staining with Coomassie Brilliant Blue R-250. The predicted molecular weight of NM is 28,500; however, due to the presence of the highly charged M region, NM migrates aberrantly by SDS–PAGE at ~45,000.

This procedure is equally effective in purifying His₁₀NM if the columns are reversed.²⁷ In this case, the Ni²⁺-Nta agarose column must be pre-equilibrated with Q elution buffer H. We prefer to use the protocol described here, however, because it is effective in removing trace metals leached from the Ni²⁺-Nta agarose resin,⁴⁹ imidazole, and carboxy-terminal truncations of the expressed protein, which affect the kinetics of fiber assembly profoundly.

**Purification of Nontagged NM**

**Buffers**

Lysis buffer N: 10 mM Tris–HCl (pH 7.2), 8 M urea  
Q wash buffer N: 10 mM Tris–HCl (pH 7.2), 8 M urea, 85 mM NaCl  
Q elution buffer N: 10 mM Tris–HCl (pH 7.2), 8 M urea, 150 mM NaCl  
HA preequilibration buffer: 10 mM Tris–HCl (pH 7.2), 8 M urea, 150 mM NaCl

HA wash buffer I: 1 mM potassium phosphate (pH 6.8), 8 M urea, 1 M NaCl
HA wash buffer II: 25 mM potassium phosphate (pH 6.8), 8 M urea
HA elution buffer: 8 M urea, 125 mM potassium phosphate

Procedure. All steps are carried out at 25°.

The precleared supernatant from cell lysis is applied to a 20-ml Q Sepharose Fast Flow column (Pharmacia) preequilibrated with lysis buffer N at a flow rate of 3 ml/min. The column is washed with 5 bed volumes of Q wash buffer N, and the protein is eluted in 3 volumes of Q elution buffer N.

The eluate from the Q Sepharose is loaded directly onto a 25-ml Macro Prep Ceramic Hydroxyapatite Type I 40-μm column (Bio-Rad) preequilibrated with HA preequilibration buffer. The column is washed with 2 bed volumes of HA wash buffer I and then with two bed volumes of HA wash buffer II. The protein is eluted using a linear gradient of potassium phosphate (pH 6.8) from 25 to 125 mM (equal volumes of HA wash buffer II and HA elution buffer). Fractions (5 ml) are analyzed by 12.5% SDS–PAGE (loading 10 μl per lane) followed by staining with Coomassie Brilliant Blue R-250. Fractions containing purified NM are pooled and concentrated using one of the following methods.

Quantitation and Yields

Sup35p is stained poorly by Coomassie Brilliant Blue G-250, which binds primarily to arginine residues. Protein determination methods based on binding to this dye, such as Bradford, are, therefore, unreliable for the quantitation of protein yields. Sup35p staining by Coomassie Brilliant Blue R-250, however, is a reliable method for detecting the protein following gel electrophoresis. We routinely determine the concentration of His10NM by the microbicinchoninic acid method (Micro-BCA; Pierce, Rockford, IL), using BSA as a standard. Alternately, we quantitate the protein concentration directly from the absorbance at 276 nm in 8 M urea using an extinction coefficient (ε) of 29,000 for NM. Typically, 50 mg of NM is obtained from a 1-liter culture.

Concentration and Storage of Purified Protein

Analysis of the amyloid assembly process by NM, discussed later, requires concentration of the protein to at least 30 mg/ml to allow ample dilution of denaturant while maintaining a sufficient protein concentration for fiber formation. This may be accomplished in multiple ways, and the method should be chosen based on the length of storage time required.
For short-term storage of NM, we routinely filter concentrate NM using Biomax Ultrafree-15 concentrators with a 10,000 molecular weight cutoff (Millipore). Column fractions containing NM are pooled and concentrated at 1500g for approximately 2.5 hr at 6°. The protein may be stored in this state for approximately 1 week at 4°.

For long-term storage, we methanol precipitate NM to remove urea and store the precipitate at −80°. Anhydrous methanol (100%) is added to eluates containing NM on ice at a ratio of 5:1. The mixture is incubated on ice for 30 min, and the precipitate is collected by centrifugation at 14,000g for 30 min at 4°. The pellet is then washed with 100% methanol (1/2 volume of supernatant) and collected by centrifugation again. The supernatant is removed, and the pellet is stored under 70% (v/v) methanol (1/2 volume of supernatant) at −80°. It is convenient to perform the precipitation in microcentrifuge tubes, as fractionating the protein after precipitation is less accurate.

Prior to use, the precipitated protein is collected by centrifugation at 14,000g for 30 min at 4°. The methanol is removed carefully, and the pellet is damp-dried under vacuum without heat for 5 min. The precipitated protein is resuspended in freshly made lysis buffer H to yield approximately a 30-mg/ml solution. The protein concentration should always be confirmed by one of the methods described previously.

**Polymerization Reactions**

The most detailed information regarding the assembly of Sup35p into amyloid has been gleaned from a study of the NM fragment. Full-length Sup35p will form amyloid in vitro, but the process is more cumbersome, as quantitative recovery of the protein in the amyloid form requires slow dialysis from denaturant (2 M stepwise decreases in urea until no denaturant remains). In addition, full-length Sup35p in an unpolymerized form binds to the diagnostic amyloid dye, Congo red, eliminating this assay from the repertoire available for monitoring amyloid formation. Therefore, our discussion of amyloid assembly here will be restricted to a characterization of His_{10}NM.

**General Considerations and Reaction Conditions.** Multiple factors influence the efficiency with which His_{10}NM will form amyloid in vitro. Among these, protein concentration and sufficient dilution from denaturant are the most crucial. We have found that polymerization reactions in the micromolar range for His_{10}NM form amyloid within a reasonable time frame (30–90 hr). In addition, we suggest at least a 100-fold dilution from denaturant into aqueous buffer, as excess denaturant slows or inhibits polymerization.
We have observed polymerization of His₁₀NM over a wide range of buffer, salt, temperature, and detergent conditions. In general, however, reactions proceed most efficiently within the pH range of 6.5–7.0 at 18°C. Molar concentrations of monovalent salt and Triton-X 100 up to 10% (v/v) do not alter the process at all, but even 0.05% (w/v) SDS is sufficient to inhibit polymerization. All of the following assays were conducted in Congo red binding buffer [CRBB: 5 mM potassium phosphate (pH 7.4), 150 mM NaCl]. For these analyses, His₁₀NM is diluted directly into CRBB with gentle vortexing to a concentration of 5 μM and is then incubated at 25°C without agitation.

Assembly of His₁₀NM into amyloid may be accelerated by several conditions. For example, the addition of 1/50 volume of His₁₀NM fibers preformed from a 5-μM solution of protein or yeast lysates from [PSI+] strains will decrease the time of fiber formation to 10–12 hr. Sonication of preformed fibers greatly increases their capacity to seed the assembly of freshly diluted His₁₀NM, further decreasing the polymerization to 2 hr. Alternately, constant gentle agitation on a roller drum (60 rpm) accelerates the assembly of His₁₀NM into amyloid to roughly 2 hr at micromolar concentrations.

Analysis of NM Amyloid Assembly

We, and others, have developed a number of tools to study the assembly of His₁₀NM into amyloid in vitro. We have monitored the assembly of this protein into an ordered amyloid by spectroscopy, dye binding, sedimentation, and microscopy. These techniques are described next.

Binding to 8-Anilino-1-naphthalenesulfonic Acid. 8-Anilino-1-naphthalenesulfonic acid (ANS; Aldrich, Milwaukee, WI) is a spectroscopic probe that exhibits low fluorescence in aqueous solutions and high fluorescence in hydrophobic environments, with a concomitant blue shift in the wavelength of maximum emission (λ_max). Folding intermediates, such as molten globules, exhibit increased ANS fluorescence relative to either denatured or fully folded proteins. ANS binding to His₁₀NM over a time course serves as a monitor of fiber assembly. Solutions of 5 μM His₁₀NM and 10 μM ANS are excited at 370 nm, and fluorescence emission is monitored between 420 and 570 nm at a 5-nm bandwidth. Surprisingly, structured His₁₀NM fibers exhibit a 10-fold increase in ANS fluorescence accompanied by a ~40-nm blue shift in the λ_max of emission to 484 nm compared to unpolymerized His₁₀NM (Fig. 4A). This increased fluorescence may indicate the presence of an exposed hydrophobic pocket(s) or groove(s) in mature His₁₀NM fibers.

Congo Red Binding. Similar to many other amyloidogenic proteins, His\textsubscript{10}NM fibers bind to the diagnostic dye, Congo red (Sigma).\textsuperscript{51} Monitoring Congo red binding over an assembly time course is a sensitive probe for fiber formation (Fig. 4C). The absorbance of a solution of 1 \( \mu M \) His\textsubscript{10}NM and 10 \( \mu M \) Congo red in CRBB is monitored between 400 and 600 nm. His\textsubscript{10}NM fibers exhibit a spectral shift in absorbance, with a new peak at 540 nm, in comparison with unpolymerized protein or Congo red alone. The amount of Congo red bound to His\textsubscript{10}NM may be calculated using the following equation:

\[
\text{mole Congo red bound/liter solution} = (A_{540}/25,295) - (A_{477}/46,306)
\]

where \( A_{540} \) and \( A_{477} \) refer to the absorbance at 540 and 477 nm, respectively.\textsuperscript{51} Under these conditions, His\textsubscript{10}NM binds roughly 4.4 moles of Congo red per mole of protein with a \( K_d \) of 250 nM.\textsuperscript{22} In addition, fibers of His\textsubscript{10}NM stained with a solution of Congo red exhibit apple-green birefringence when viewed by polarized light.\textsuperscript{23}

Special consideration should be given to maintain identical buffer conditions when comparing different samples, as the quantity of Congo red binding to proteins is altered by pH, denaturant, and metals.\textsuperscript{27}

**SDS Solubility.** Assembly of His\textsubscript{10}NM into amyloid may also be monitored by the degree of solubilization in 2\% (w/v) SDS. Unpolymerized protein remains soluble in 2\% (w/v) SDS. In contrast, once amyloid has formed, these structures are largely insoluble in 2\% SDS at room temperature. We have utilized this difference in SDS solubility, combined with SDS–PAGE, as an assay to monitor fiber formation. SDS sample buffer is added to a 1\times concentration to two 20-\mu l aliquots of a 5 \mu M polymerization reaction. One sample is boiled in a water bath for 10 min, whereas the other sample is incubated at room temperature. The samples are then separated on a 10\% SDS–PAGE gel that is subsequently stained with Coomassie Brilliant Blue R-250 (Fig. 5A). The same amount of unpolymerized His\textsubscript{10}NM enters the gel whether or not the sample has been boiled. In contrast, His\textsubscript{10}NM fibers only enter the gel in boiled samples.

**Limited Proteolysis of NM.** Limited proteolysis of His\textsubscript{10}NM with chymotrypsin and V8 provides sensitive probes for domain-specific structural changes during amyloid assembly. The N region contains 20 tyrosine residues, which are high-affinity sites for cleavage with the protease chymotrypsin, whereas the M region contains none. Conversely, the M region contains 23 glutamic acids, which are high-affinity sites for cleavage with V8 protease, whereas the N region contains none. Alterations in the digestion pattern reflect either a change in conformation or accessibility for either the N (chymotrypsin) or the M (V8) region.

New batches of proteases should be titrated with known samples (both fibers and freshly diluted His\textsubscript{10}NM). Samples (20 \mu l) of a 5 \mu M solution of NM in CRBB are incubated with either chymotrypsin (\textasciitilde 1/250, w/w) or V8 (\textasciitilde 1/25, w/w) at 37\°C for 15 min. Proteases are freshly resuspended at a concentration of 1 mg/ml in 1 mM HCl for chymotrypsin (Boehringer Mannheim) or 1 mg/ml in water for V8 (Endoproteinase Glu-C; Boehringer Mannheim). The reaction is terminated by adding SDS sample buffer to 1\times and boiling for 10 min in a water bath to inactivate proteases. Digestion products are then separated on 10\% SDS–PAGE gels that are subsequently stained with Coomassie Brilliant Blue R-250. V8 cleavage of His\textsubscript{10}NM fibers produces a characteristic digestion pattern that is distinct from that of unpolymerized His\textsubscript{10}NM (Fig. 5B). In contrast, His\textsubscript{10}NM fibers exhibit resistance to chymotrypsin digestion (Fig. 5C), whereas unpolymerized His\textsubscript{10}NM is cleaved rapidly.

**Sedimentation Analysis.** Another convenient assay for monitoring His\textsubscript{10}NM assembly is differential sedimentation. In contrast to unpolymer-
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**Fig. 5.** Biochemical analysis of His-tagged NM amyloid fibers. (A) SDS solubility. Coomassie Brilliant Blue-stained 10% SDS–polyacrylamide gels of unpolymerized His-tagged NM (Initial; lanes 1 and 2) or amyloid (Fibers; lanes 3 and 4) incubated in 2% (w/v) SDS without (−) or with (+) boiling are shown. (B and C) Limited proteolysis with V8 and chymotrypsin, respectively. Unpolymerized His-tagged NM (Initial) or amyloid (Fibers) were digested with V8 protease (B) or chymotrypsin (C) and separated on a 10% SDS–polyacrylamide gel that was subsequently stained with Coomassie Brilliant Blue. His-tagged NM incubated in the absence of protease—is shown for comparison. The positions of molecular weight standards are indicated at the right.
ized His$_{10}$-NM, His$_{10}$-NM fibers will sediment at high speeds. Samples are centrifuged at 100,000g for 10 min at 4°C and are then analyzed by 10% SDS-PAGE. The supernatant is removed following centrifugation, and SDS sample buffer is added to 1×. An equal volume of 1× SDS sample buffer is added to the pellet. Both samples are boiled for 10 min in a water bath and loaded onto a 10% SDS-PAGE, which is subsequently stained with Coomassie Brilliant Blue or transferred to Immobilon-P (Millipore) for quantitative Western blot analysis using $^{125}$I-labeled protein A (Amer- sham). Partitioning between the supernatant and the pellet fractions is indicative of the assembly state, unpolymerized or polymerized, respectively.

Alternately, His$_{10}$-NM assembly may be monitored using radiolabeled protein. His$_{10}$-NM is radiolabeled with $^{[35]}$S methionine, purified, and added to a polymerization reaction (10,000 cpm/50 µl of reaction, supplemented with unlabeled NM to 5 µM). Samples (50 µl) are removed and separated into supernatant and pellet fractions as described earlier for unlabeled protein. Following centrifugation, the soluble counts remaining in the supernatant are measured in a scintillation counter as an indication of the extent of the reaction.

The labeling procedure follows, and the protein is purified by one of the methods described previously.

1. Grow a single colony of BL21 [DE3] pAP lacI$^q$ harboring the expression plasmid to an OD$_{600}$ of 0.2 at 37°C at 300 rpm in 1 liter of Circle Grow medium (Bio 101) supplemented with 50 µg/ml kanamycin and 200 µg/ml ampicillin.
2. Collect the cells by centrifugation at 1500g for 10 min at 4°C.
3. Resuspend the pellet in 1 liter of M9 medium (with MgCl$_2$ substituted for MgSO$_4$) supplemented with antibiotics as described earlier. Incubate at 37°C, 300 rpm for 1 hr.
4. Collect the cells by centrifugation, as described previously.
5. Resuspend the pellet in 50 ml of M9 (with MgCl$_2$) supplemented with antibiotics as described earlier. Add 3.5 mCi of Tran $^{35}$S label (NEN-Dupont, Wilmington, DE) and IPTG to 1 mM. Incubate at 37°C, 300 rpm for 2 hr.
6. Collect cells by centrifugation as described earlier and store at −80°C or proceed with purification. A typical specific activity is 2–3 × $10^4$ cpm/µg protein, with a total yield of roughly 10 µg.

**Electron Microscopy of NM**

The most important method, to date, for identifying the presence of amyloid is by microscopy. We have utilized transmission electron micros-
Fig. 6. Electron microscopy of fibers formed by His-tagged NMC and His-tagged NM. Protein samples were prepared for visualization by electron microscopy as described. (A) A field of His-tagged NMC incubated in a high salt buffer consisting of 30 mM Tris–HCl (pH 8.0), 1.2 M NaCl, 10 mM MgCl₂, 2 mM GTP, 280 mM imidazole, and 5 mM 2-mercaptoethanol. The base fiber structure has an approximate diameter of 10.6 ± 1.0 nm and displays an amorphous structure along its side, presumably the C domain. (B) A field of His-tagged NM dissolved in 20 mM Tris–HCl (pH 8.0), 150 mM NaCl, and 4 M urea and then diluted into 20 mM Tris–HCl (pH 8.0); 1.2 M NaCl. Fibers from His-tagged NM exhibit a smooth appearance, whose approximate average diameter is 11.5 ± 1.5 nm. Scale bar: 200 nm.
copy (TEM), scanning transmission electron microscopy (STEM), and atomic force microscopy (AFM) to monitor the assembly of NM into amyloid. Although each of these techniques provides distinct information about the structure and size of complexes formed by His\textsubscript{10}NM, our discussion here will be limited to EM due to the general accessibility of this technique.

We routinely negatively stain His\textsubscript{10}NM fibers\textsuperscript{52} for EM analysis. Protein (5 \(\mu\)l of a 5 \(\mu\)M solution) is applied to a glow-discharged 400 mesh carbon-coated copper grid (Ted Pella, Redding, CA). Protein is allowed to absorb to the grid for 30 sec and is then immediately stained with 200 \(\mu\)l of 2% (w/v) aqueous uranyl acetate. Excess liquid is removed from the grid with a filter paper wick, and they are then allowed to air dry. Samples are observed in a Philips (Eindhoven, The Netherlands) CM 120 transmission electron microscope at an accelerating voltage of 120 kV in low-dose mode. Samples are viewed at a magnification of 40,000\( \times\), and images are recorded on Kodak (Rochester, NY) SO 163 film.

Fibers formed by His\textsubscript{10}NM (Fig. 6A) have an apparent average diameter of 11.5 \(\pm\) 1.5 nm.\textsuperscript{22} The structure of fibers formed from His\textsubscript{10}Sup35p is sensitive to buffer conditions. Fibers formed in moderate ionic strength buffer (20 mM potassium phosphate (pH 7.5), 20 mM KCl, 5 mM MgCl\textsubscript{2}, 2.5 mM 2-mercaptoethanol) are smooth and have an average diameter of 17 \(\pm\) 2.0 nm.\textsuperscript{22} In high ionic strength buffer (Fig. 6B), fibers of full-length His\textsubscript{10}Sup35p are more extended, revealing an interior rod (diameter 10.6 \(\pm\) 1.0 nm) and an amorphous outer layer.

Conclusion

The study of amyloidogenic proteins is complex both \textit{in vivo} and \textit{in vitro}. Each assay presented in this article provides unique information about the physical state of Sup35p, but these techniques also have inherent pitfalls. We suggest that a robust characterization of transitions in Sup35p physical states both \textit{in vivo} and \textit{in vitro} requires analysis with a combination of techniques.

Acknowledgments

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