Analysis of the Generation and Segregation of Propagons: Entities That Propagate the \([PSI^+]\) Prion in Yeast

Brian Cox, Frederique Ness and Mick Tuite

Research School of Biosciences, University of Kent, Canterbury, Kent CT2 7NJ, United Kingdom

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ABSTRACT

The propagation of the prion form of the yeast Sup35p protein, the so-called \([PSI^+]\) determinant, involves the generation and partition of a small number of particulate determinants that we propose calling “propagons.” The numbers of propagons in \([PSI^+]\) cells can be inferred from the kinetics of elimination of \([PSI^+]\) during growth in the presence of a low concentration of guanidine hydrochloride (GdnHCl). Using this and an alternative method of counting the numbers of propagons, we demonstrate considerable clonal variation in the apparent numbers of propagons between different \([PSI^+]\) yeast strains, between different cultures of the same \([PSI^+]\) yeast strain, and between different cells of the same \([PSI^+]\) culture. We provide further evidence that propagon generation is blocked by growth in GdnHCl and that it is largely confined to the S phase of the cell cycle. In addition, we show that at low propagon number there is a bias toward retention of propagons in mother cells and that production of new propagons is very rapid when cells with depleted numbers of propagons are rescued into normal growth medium. The implications of our findings with respect to yeast prion propagation mechanisms are discussed.

THE \([PSI^+]\) factor of Saccharomyces cerevisiae is inherited in an extrachromosomal fashion and is propagated by a prion-like mechanism similar to that proposed for the infectious properties of mammalian transmissible spongiform encephalopathies (TSEs; Wickner 1994). There is now considerable genetic and biochemical evidence to support the prion theory for \([PSI^+]\) (see Wickner and Chernoff 1999; Serio and Lindquist 1999, 2000 for recent reviews). The prion protein involved, Sup35p (also called eRF3), is essential for the propagation of the \([PSI^+]\) phenotype; for example, various \(SUP35\) gene mutations in the region encoding the N terminus of Sup35p affect the transmission of the \([PSI^+]\) phenotype (Ter-Avanesyan et al. 1993; Doel et al. 1994; De Pace et al. 1998; Liu and Lindquist 1999; Parham et al. 2001). Furthermore, Sup35p forms amyloid-like fibrils \textit{in vitro}, especially if seeded with material from a \([PSI^+]\) strain (Paushkin et al. 1997; De Pace et al. 1998; Serio et al. 2000), while \textit{in vivo}, much of the Sup35p in \([PSI^+]\) cells sediments in the S100-pelletable fraction of cell-free lysates (Patino et al. 1996; Paushkin et al. 1996). Yet little is known about the mode of propagation of the \([PSI^+]\) prion.

By analogy with PrP of mammals (Prusiner et al. 1998), Sup35p must exist in two forms, let us say \(\alpha\) (normal) and \(\beta\) (prion), and the presence of the \(\beta\)-form is sufficient to convert molecules in the \(\alpha\)-form to \(\beta\), which condenses into sedimentable high molecular weight aggregates or forms amyloid fibrils. The sequestration of aggregates in its \(\beta\)-form and concomitant depletion of the functional \(\alpha\) form are held to be responsible for the \([PSI^+]\) phenotype, namely enhanced suppression of nonsense mutations (reviewed in Serio and Lindquist 1999). However, it remains to be established whether any of this is true in living cells. For instance, it is not known what—if any—process is involved in converting \(\alpha\) to \(\beta\) or whether special processes are involved in formation of aggregate/amyloid fibers from \(\beta\)-form Sup35p, whether amyloid itself is sufficient to promote \(\alpha\) to \(\beta\) conversion, or whether some intermediate stage or complex is involved. In particular, it is not known how the ability to maintain \([PSI^+]\) is passed from mother to daughter cells to promote stable inheritance. It may depend on a random diffusion of prions between mother and daughter cells at cell division; a propagating module may be replicated in synchrony with cell division, or there may be a cycle of accretion to, and degradation of, Sup35p-containing aggregates.

There are several lines of evidence that \([PSI^+]\) inheritance is particular. For example, when newly formed zygotes between \([PSI^+]\) and \([psi^-]\) cells are sporulated, the spores that are formed either give rise to clonally pure \([PSI^+]\) and prion-free \([psi^-]\) colonies or give rise to sectored colonies, without intermediate phenotypes (Young and Cox 1971). However, the clearest evidence that the inheritance of \([PSI^+]\) is particular has come from studies on the kinetics of \([PSI^+]\) elimination when propagation of the \([PSI^+]\) determinant is blocked using
low concentrations (1–3 mM) of guanidine hydrochloride (GdnHCl; Tutte et al. 1981; Eaglestone et al. 2000; Ferreira et al. 2001; Jung and Masison 2001).

These studies led to four main conclusions: (a) the cells behaved as if they normally contained a certain number of \([PSI^+]\) “seeds”, (b) these numbers ceased to increase while the cells were growing in GdnHCl, so that the average number per cell was halved at each generation regardless of the rate of growth, (c) segregation of the remaining seeds was completely random at each cell division, and (d) propagation of \([PSI^+]\) was restored in any cell containing one or more seeds immediately on return to medium lacking GdnHCl. Furthermore, the numbers of cells having the ability to grow into \([PSI^+]\) cultures reaches a constant level during continued growth in the presence of GdnHCl (Cox 1993). If a culture goes into stationary phase before curing of \([PSI^+]\) is complete, the numbers of \([PSI^+]\) cells remain constant, suggesting that GdnHCl does not destroy \([PSI^+]\) seeds, but merely prevents their replication. If this model is correct, one can predict \(N_0\), the initial number of \([PSI^+]\) particles per cell, from the number of generations it takes to “cure” the culture. Eaglestone et al. (2000) presented a mathematical model based on a random binomial distribution of particles at cell division, which arrived at a number for \(N_0\) of 62.

Here we address four questions arising from the studies of Eaglestone et al. (2000):

1. Is the value \(N_0 = 62\) constant or peculiar to the strain of yeast and/or the culture conditions used?
2. Eaglestone et al. (2000) showed that on transfer of a partially “cured” culture, from GdnHCl to ordinary growth medium, no further segregation of \([psi^-]\) cells was observed, although with only a few particles remaining per cell, random segregation should have caused further segregation. We have considered two ways of accounting for this: (a) the recovery of a sufficiently high number of seeds occurs very rapidly on return to normal growth medium, taking less than one generation, or (b) individual seeds are segregated by an independent physical structure so that they enjoy a nonrandom segregation, but the number of the segregating structures carrying \([PSI^+]\) particles is reduced by the lack of \([PSI^+]\) generation in the presence of GdnHCl. This would give the appearance of random segregation during growth in GdnHCl medium, until cells were returned to normal medium when their replication and directed segregation would stop further loss of seeds.
3. Do \([PSI^+]\) seeds and the cells that contain them survive GdnHCl treatment indefinitely?
4. Do \([PSI^+]\) seeds segregate at random?

Normally, prion “aggregates” or “fibers” are assayed by methods that measure amounts of material, but not the numbers of such entities. However, we have no information about the structure of these materials in vivo nor about whether they are the entities that propagate the \([PSI^+]\) phenotype. To maintain a distinction between aggregates or fibers and entities that propagate the \([PSI^+]\) phenotype, we propose that the latter be referred to as “propagons.”

MATERIALS AND METHODS

**Yeast strains:** The following strains of *S. cerevisiae* were used in this study:

- BSC783/4a, MATα, ade2-1\(^{UAA}\), his3-11, -15, leu2-3, -119, ural-3, ltrp1, [PSI\(^+\)];
- BSC783/4c, MATα, ade2-1\(^{UAA}\), his3-11, -15, leu2-3, -119, ural-3, ltrp1, [PSI\(^+\)];
- BSC772/18c, MATa, ade2-1\(^{UAA}\), his4-712, leu2-3, -119, ural-1, SUQ5, [PSI\(^+\)];
- 74-D694, MATa, ade1\(^{UCA}\), his3-11, -15, leu2-3, -119, ural3, trp1, [PSI\(^+\)] (strong) or (weak);
- BSC783/4a × BSC772/18c [PSI\(^+\)], diploid strain;
- BSC783/4a × BSC783/4c [PSI\(^+\)], diploid strain.

**Growth media:** General growth medium was YPD: 1% peptone, 1% yeast extract, 2% dextrose. For distinguishing [PSI\(^+\)] from [psi\(^-\)] colonies growing on solid medium, 1/\(3\)YE medium (0.25% yeast extract, 1% peptone, 4% dextrose) was used. On 1/\(3\)YE medium, ade1 or ade2 adenine-requiring colonies are deep red while adenine-independent colonies are white. Synthetic medium for selection of prototrophs was based on 0.67% yeast nitrogen base (YNB) without amino acids (Difco, Detroit), 2% in dextrose with appropriate supplements (Kaiser et al. 1994).

**Cell plating and counting:** The methods used were those essentially described by Eaglestone et al. (2000), except that the constant small numbers of colonies sectored red ([psi\(^-\)]) and white ([PSI\(^+\)]) were not counted as coming from two cells but as derived from one [PSI\(^+\)] cell. This is because, for the purposes of calculation, the relevant numbers are those of cells plated of the \(p_0\) class, i.e., having no [PSI\(^+\)] propagons. We make the assumption that sectored colonies can arise only from cells that contained at least one propagon at the time of plating onto the agar.

**Calculating \(N_0\), the number of propagons per cell:** The model for \([PSI^+]\) segregation presented by Eaglestone et al. (2000) is based on an iteration of a binomial distribution of random numbers of propagons at each generation and requires a unique computer program. The same predictions can be obtained by using the Poisson formula for random distribution of small numbers of objects or events in defined blocks of space or time (Figure 1). In our experimental system, the blocks of space are yeast cells and the “objects” are the propagons. This formula gives the result that when a random distribution is such that there is, on average, one propagon per cell (for example) there will be 36.8% of the cells in the \(p_0\), i.e., [psi\(^-\)] class. Thus, if the data are plotted as the percentage of [PSI\(^+\)] cells as a function of generations of growth in GdnHCl medium, when 63.2% is reached, \(N_0\) can be found from the value of \(2^g\), where \(g\) is the number of generations elapsed at that moment, assuming that the propagon number halves at each generation. Indeed, \(N_0\) can be found from any value of \(p_0\) (the fraction of [psi\(^-\)] colonies), using \(p_0 = e^{-m}\) to find \(m\), the mean number of entities per cell at that time, and multiplying by \(2^g\).

**Sampling whole colonies:** Cells for phenotypic analysis were plated at a density of ~100 per agar plate and, after 48–72 hr incubation, individual colonies were cut from the plate.
with clonally pure cultures of the BSC783/4a \([PSI^+]\) strain maintained in logarithmic growth in YPD + 3 mM GdnHCl, as a function of numbers of generations. Theoretical curves are plotted using the Poisson distribution for estimating the percentages of \([PSI^+]\) cells (see MATERIALS AND METHODS) on the assumption that no generation or destruction of \([PSI^+]\) propagons takes place in these conditions and that the average number of propagons per cell therefore halves at each generation. The curves shown were plotted assuming initial average numbers \(N_0\) of propagons of 32, 256, or 1024 for the different cultures. There is therefore significant clonal variation in the number of \([PSI^+]\) propagons for a given \([PSI^+]\) yeast strain.

To determine whether or not such clonal variation was strain specific, we extended this analysis to other isolates of BSC783/4a \([PSI^+]\) and various other haploid and diploid \([PSI^+]\) strains (Table 1). Most GdnHCl-treated cultures gave a profile of \([psi^-]\) segregation that fell closely along theoretical curves. The data in Table 1 further illustrate a lack of consistency of estimated numbers of propagons, even among different cultures of the same strain.

**Generation of propagons after release from a guanidine hydrochloride block:** To determine how quickly propagons are multiplied when cells are returned to normal YPD medium from YPD + GdnHCl medium, a culture of BSC783/4c \([PSI^+]\) was transferred during log phase into YPD + 3 mM GdnHCl medium and samples were taken at various intervals. After seven generations of growth, part of the culture was transferred to fresh YEPD without GdnHCl and samples were taken from both the culture still in YPD + GdnHCl (G0) and the “rescued” one, R. At intervals from this time, portions

### Table 1

<table>
<thead>
<tr>
<th>([PSI^+]) strain/isolate</th>
<th>Estimated propagon no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haploids</td>
<td></td>
</tr>
<tr>
<td>BSC783/4a, 0(^*)</td>
<td>32</td>
</tr>
<tr>
<td>BSC783/4a, 3</td>
<td>128</td>
</tr>
<tr>
<td>BSC783/4a, 4</td>
<td>128</td>
</tr>
<tr>
<td>BSC783/4a, G(_0)</td>
<td>215</td>
</tr>
<tr>
<td>BSC783/4a, 0, 2(^*)</td>
<td>891</td>
</tr>
<tr>
<td>BSC783/4c</td>
<td>388</td>
</tr>
<tr>
<td>74-D694 (strong)</td>
<td>128</td>
</tr>
<tr>
<td>Diploids</td>
<td></td>
</tr>
<tr>
<td>BSC783/4a × BSC783/4c, 2(n)</td>
<td>128</td>
</tr>
<tr>
<td>BSC783/4a × BSC783/4c, 2(n)</td>
<td>588</td>
</tr>
</tbody>
</table>

The number of propagons was calculated as described by Eaglestone et al. (2000).

\(^*\) Shown in Figure 1.

\(^\dagger\) Shown in Figure 2.

#### RESULTS

**Clonal variation in the numbers of \([PSI^+]\) propagons:**
Figure 1 shows the decrease in percentage of \([PSI^+]\) colonies recovered from three different experiments with propagons in any given cell, \(p_n\) is the fraction of cells giving rise to \([psi^-]\) colonies (when \(n = 0\) in those cells), and \(m\) is the average number of \([PSI^+]\) propagons per cell at any given time. Each curve starts at a different average number of propagons \((N_0 = m)\) and assumes a halving of this number at each generation. Perpendiculars have been drawn from the 63.2% point on each curve. This is the point at which there is, on average, one propagon per cell, which allows calculation of \(N_0\), the initial value of \(m\), from the number of generations elapsed.

<table>
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The number of propagons was calculated as described by Eaglestone et al. (2000).

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\(^\dagger\) Shown in Figure 2.

Micromanipulation: Use was made of a Singer MSM System from Singer Instruments (Watchet, United Kingdom), to transfer individual cells and to separate mother and daughter cells.
of R were returned to GdnHCl medium, designated G1–G4, and samples were taken for plating as with the conversion event. To test this hypothesis we sampled other cultures (Figure 2A). The curing curves for all whole colonies taken from YPD medium (hours in parentheses) were: G0, 0 hr, ×; R, 12.5 hr (0), ■; G1, 13.25 hr (0.75), □; G2, 14 hr (1.5), △; G3, 14.75 hr (2.25), ○; G4, 15.75 hr (3.25), ◊. (B) Data of A replotted to normalize all cultures to the time at which they were put into or returned to GdnHCl medium and to 100% [PSI+] colonies at each time point. Percentages have been calculated from hemocytometer and colony counts allowed to grow into colonies, every colony should contain a single [psi−] cell, yielding between 20 and 150 [PSI+] colonies, with varying numbers of other types of nuclear Ade+ revertants (Figure 4, A and C). The distributions of numbers are not strictly Gaussian as would be expected from a random distribution of propagon numbers among the cells of the [PSI+] population, with the rest having none and at the time of transfer to GdnHCl has been subtracted from being [psi−] as determined following plating on 1⁄4YE from a culture of BSC783/4a shown in Figure 3 was plotted. The kinetics of the increase in N0 with time was exponential for the first 2 hr with a doubling time of ~20 min, as we have previously reported (Ness et al. 2002). The N0 for G0, the parent culture, and for G4, which spent the longest time in YPD before return to GdnHCl medium, was 215 (7.75 generations to 63% [PSI+]).

An alternative method for estimating the number of [PSI+] propagons in a strain: If a cell starts in GdnHCl medium with n propagons, then after several generations of growth, the n propagons will be segregated into n cells of the population, with the rest having none and being [psi−] (Eaglestone et al. 2000). Thus, if [PSI+] cells are plated on a GdnHCl-containing medium and allowed to grow into colonies, every colony should contain n cells with a propagon each. Conversely, the [psi−] colonies that grow on YPD from single [psi−] cells should have no cells containing propagons apart from those that may be generated by a spontaneous de novo conversion event. To test this hypothesis we sampled whole colonies taken from YPD + GdnHCl plates that had grown from [PSI+] cells and whole colonies grown on YPD from [psi−] cells. The results from two otherwise isogenic [PSI+]/[psi−] pairs are presented in Figure 4.

Every colony sampled from the 1⁄4YE + GdnHCl plates, i.e., which had started from a [PSI+] cell, yielded between 20 and 150 [PSI+] colonies, with varying numbers of other types of nuclear Ade+ revertants (Figure 4, A and C). The distributions of numbers are not strictly Gaussian as would be expected from a random distribution of propagon numbers among the cells of the [PSI+]...
Yeast Prions and Propagons

Figure 4.—The numbers of \([PSI^+]/H11001\) (solid bars) and nuclear \(Ade^+/H11001\) (open bars) revertants recovered from two different \([PSI^+]/\) strains growing in the presence of 3 mm GdnHCl. The nuclear mutation rate was calculated from the \(p_0\) value. (A) BSC783/4a \([PSI^+]/\) growing on YPD + 3 mm GdnHCl plates. In the original culture giving rise to these colonies, 9.3% of cells had no bud, 68.3% had one bud, 19.7% had two buds, and 2.7% had three or more buds. \([PSI^+]/\) colony is an average and the nuclear mutation rate was calculated from the \(p_0\) value (Lea and Coulson 1949). (B) BSC783/4a \([psi^-]/\) colonies taken from YPD plates. The data are pooled from two experiments and both the \([psi^-]/\) and nuclear mutation rates are calculated from the \(p_0\) values. (C) 74-D694 \([PSI^+]/\) growing on YPD + 3 mm GdnHCl plates. The nuclear mutation rate was estimated from the median value of numbers recovered from the set of colonies sampled, since there was no \(p_0\) class. \([PSI^+]/\) colony is an average. In the culture giving rise to these colonies, 3.1% of cells had no bud, 71.8% had one bud, 0% had two buds, and 25% had three or more buds. There were several large “clumps” of cells characteristic of this strain when grown in liquid YEPD medium. (D) 74-D694 \([psi^-]/\) growing on YEPD. The \([PSI^+]/\) mutation rate was estimated from the \(p_0\) value and the nuclear mutation rate from the median value of numbers found in the sampled colonies.

colony taken for plating; in fact, in each experiment there is a small number of “jackpots.” The most important observation is that, as predicted, there were no colonies from which no \([PSI^+]/\) revertants could be recovered.

In the colonies started from \([psi^-]/\) cells the numbers of \([PSI^+]/\) revertants showed the fluctuation typical of random reversions occurring in growing cultures (Luria and Delbruck 1943; Lea and Coulson 1949), with about one-third of the colonies yielding no \([PSI^+]/\) revertants at all (Figure 4, B and D). As expected, the nuclear \(Ade^+/\) revertants also showed this type of fluctuation pattern, which is typical of random spontaneous mutation, in both sets of colonies: those from \([PSI^+]/\) cells plated on \(\frac{1}{2}YE + GdnHCl\) medium and those from \([psi^-]/\) cells growing on YPD medium. Approximate mutation rates could be calculated from these fluctuation tests from the \(p_0\) values or median values. The number of novel mutations recovered from a colony is very sensitive to the total number of cells in the colony since most mutations occur in the last one or two cell divisions before sampling and colony size could be controlled only approximately in the course of sampling. This is not true of the numbers of \([PSI^+]/\) cells found in the
Results were similar, with "weak" [PSI⁺] variants of the strain 74-D694, which differ in both the extent of the [PSI⁺] nonsense suppression phenotype (Derkatch et al. 1997) and the level of soluble Sup35p (Uptain et al. 2001). The results were essentially similar to those obtained with the strain BSC783/4a [PSI⁺] (Figure 4, A and B) and hence the results were pooled (Figure 4, C and D). We also repeated the control experiment using colonies grown from [psi⁻] cells plated on YPD + 3 mM GdnHCl. Results were similar, with [PSI⁺] revertants appearing at a rate estimated from the p₀ value of 5 × 10⁻⁷. There were 3 colonies with zero [PSI⁺], 29 with numbers between 1 and 10, 11 between 11 and 20, and 2 with jackpots of >50.

To confirm that the number of [PSI⁺] colonies was independent of colony size, we sampled one set of colonies that grew when they had reached a size of ~1000 cells per colony (after 24 hr and 10 generations) and a second set after 72 hr when there were 1–2 × 10⁶ cells per colony (~26 generations of growth). The first set yielded 141 [PSI⁺] colonies per colony sampled and the second 145 [PSI⁺] per colony sampled.

Our data are consistent with the prediction that [PSI⁺] propagons exist as physical entities that, when their generation is blocked, segregate eventually into a few cells in the culture (Eagleton et al. 2000). Furthermore, the conversion of the [PSI⁺] cells into [psi⁻] during growth in GdnHCl is not due to the destruction of propagons. In both strains examined here, no or very few [PSI⁺] cells were recovered from [psi⁻] colonies growing on YPD, and in both strains generally between 20 and 100 [PSI⁺] cells were recovered from colonies grown from [PSI⁺] cells plated onto 3 mM GdnHCl-containing medium; among these, no colonies were found without any [PSI⁺] cells.

The average number of [PSI⁺] propagons found by our alternative method is less than that normally estimated by the kinetics of the "curing" experiment. This discrepancy is probably due to a <100% recovery of every potential Ade² [PSI⁺] cell from each colony. With only one propagon in the cell, the diagnostic suppressor phenotype of the [PSI⁺] cell may appear only after considerable delay. In subsequent experiments, described below, a preincubation was carried out.

The non-Gaussian distributions with a very high variance observed in numbers of [PSI⁺] cells recovered from different colonies may be due to the considerable degree of variation in the size of colony-forming units, particularly in the 74-D694 strains (Figure 4). This would have the effect that different colonies would arise from different numbers of cells: one, two, three, or more than three. Thus each colony would start from an inoculum containing some multiple of the average number of propagons per cell, and this would be the number of [PSI⁺] colonies recovered from it at the end of the period of growth.

**The timing of generation of [PSI⁺] propagons:** Instead of sampling random colonies, colonies were grown from individual cells isolated using a micromanipulator. From a log-phase culture of 74-D694 [PSI⁺] (strong) growing in YPD at a titer of 5 × 10⁶/ml, cells were chosen that were: (1) unbudded, i.e., G₁ phase; (2) with a small bud, i.e., S phase; (3) with a large bud, i.e., G₂ phase; or (4) three-cell clusters with the mother cell carrying a very small bud and the daughter cell as yet unbudded, i.e., S + G₁ phases. Approximately 40 of each type of cell were taken from a log-phase culture freshly placed on 1/4YE medium containing 3 mM GdnHCl. Each
The inhibition of generation of new propagons occurs mainly in S phase of the cell cycle. The average numbers of [PSI+] cells found in colonies derived from various cell types isolated by micromanipulation from [PSI+] cultures and placed on 1/4YE + 3 mM GdnHCl medium are shown. These data are taken from the experiment shown in Figure 5. The averages are plotted against an idealized cell cycle arbitrarily assigning a cycle time of 2 hr (Pringle and Hartwell 1981).

The numbers of [PSI+] cells recovered from the colonies grown from G1- or S-phase cells form a distribution about means of 40.8 and 31.3, respectively (Figure 5). The G2 cells and three-cell clusters gave colonies with mean numbers of [PSI+] cells of 103.2 and 75.0, respectively. However, clearly a great deal of variation in numbers was found in these colonies, with a few yielding high numbers: for example, one yielded 880 [PSI+] colonies and three yielded >200. In calculating these means the colonies yielding such clear “jackpot” events were disregarded. The three-cell clusters used were considered to be pairs of G1- and S-phase cells and the sum of the means of the latter distributions, i.e., 72.1 (40.8 + 31.3), is similar to the mean of the former, i.e., 75.0. We take this as a validation of this method of estimating propagon numbers, with the reservation that some explanation must be found for the jackpots.

The increase in propagon numbers (and possibly in variance) was confined to the stage between small-bud formation (initiation) and growth into a large bud. This coincides with the S phase of the cell cycle with this phase lasting for ∼25% of the cell cycle (Pringle and Hartwell 1981). No further apparent increase occurred after this while M and G2 (also ∼25% of the cycle) were taking place or during G1 since the numbers of propagons recovered from large-bud cells and three-cell clusters were about the same (Figure 6).

Inhibition of [PSI+] propagon generation by guanidine hydrochloride: The inhibition of generation of propagons is an inference drawn from the kinetics of curing during growth in GdnHCl. We tested this by micromanipulating cells from a [PSI+] culture growing in YPD + 3 mM GdnHCl medium, choosing samples of unbudded cells from the culture at the time of addition of GdnHCl and intervals of approximately one generation thereafter. The cells were picked onto fresh 1/4YE + 3 mM GdnHCl. Throughout the experiment cell number was determined using a hemocytometer and the effectiveness of the medium in curing [PSI+] was confirmed by plating out cells from the culture onto 1/4YE after 10 generations of growth. If the “inhibition of generation” hypothesis is correct, at each generation the average number of propagons per cell would be halved. The results (Table 2) showed an exponential decline in numbers of [PSI+] cells found in successive samples of the culture, which extrapolates back to the origin. The halving time for the decline was ∼2.5 hr, slightly slower than the rate at which the cell numbers doubled in the culture that was measured at 2.2 hr.

The randomness of propagon segregation between mother and daughter: Eaglestone et al. (2000) proposed that propagons distribute randomly between mother and daughter cell during cytokinesis. We set out to validate this experimentally in two ways. First, in the course of the experiment described above, in addition to sampling unbudded cells, three-cell clusters were taken and the mother (small-budded) and daughter (unbudded) separated on 1/4YE + 3 mM GdnHCl medium. If GdnHCl does inhibit propagon generation, then any differences between mothers and daughters should reflect a segregation bias. Cells were taken from cultures after 4 and 6 hr in GdnHCl and the resulting colonies were assayed for the presence of [PSI+] cells (Figure 7A). The data are consistent with there being a bias toward propagons remaining in the mother cell, with a daughter:mother ratio of 1:2 being observed after both 4- and 6-hr incubation in GdnHCl.

Biased propagon segregation was also found in a [PSI+] culture growing in YPD (Figure 7B) as defined using the same experimental strategy described above. Not all mother-daughter pairs showed the bias (and in one case it was reversed) although the bias could be accounted for by propagon generation having taken

<table>
<thead>
<tr>
<th>Hours after addition of GdnHCl</th>
<th>No. of cells sampled</th>
<th>Range of nos. of [PSI+] cells in each colony</th>
<th>Average</th>
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<tr>
<td>0</td>
<td>18</td>
<td>30–228</td>
<td>114.3</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>31–184</td>
<td>62.7</td>
</tr>
<tr>
<td>4</td>
<td>7</td>
<td>8–93</td>
<td>35.6</td>
</tr>
</tbody>
</table>

The single-cell isolates were sampled by micromanipulation from the surface of YPD + 3 mM GdnHCl plates and grown into colonies as described in the text.

![Figure 6](image-url)
ters, and some propagon generation might indeed occur after the cells are exposed to GdnHCl.

In a second approach cells were taken from \([\psi^+]/H11001\) cultures growing in YPD + GdnHCl medium at a stage when, after several generations there should be, on average, only one or two propagons per cell. The overall fraction of \([\psi^-]\) cells in the cultures was determined and at the same time, suspensions of these cultures were transferred to fresh \(\frac{1}{2}YE\) medium and cells micromanipulated to marked positions on the agar surface. In the first experiment (BSC783/4a; nos. 1 and 2 in Table 3) large-budded cells were separated with the larger of each pair being identified as the mother. In subsequent experiments, three-cell clusters were taken to separate, identifying the budded member of each pair as the mother since mother cells reach the size required for bud initiation before their daughters (Pringle and Hartwell 1981). When \([\psi^+]\) cells have been growing in GdnHCl for 14–18 hr and have undergone several generations of division, there is a strong bias in propagon segregation with the propagons remaining with the mother cell in \(\approx 90\%\) of the cases (Table 3).

**DISCUSSION**

**Counting yeast propagons:** Current data are consistent with the propagation of the \([\psi^+]\) prion determinant in yeast involving a number of replicating “seeds” that are transmitted through the cytoplasm to daughter cells at cytokinesis. We have termed these seeding elements propagons and they are most likely composed of aggregates of nonnative conformers of the Sup35p protein.

**Inhibition of propagon replication by guanidine hydrochloride:** The numbers of propagons in cells growing in \(3\,\text{mm}\,\text{GdnHCl}\) halved every 2.5 hr from the time of addition of the GdnHCl. This was slightly slower than the doubling time of the culture (\(\approx 2.2\) hr) and raises the possibility that generation of propagons is not completely inhibited by growth in GdnHCl. This would affect our method of estimating propagon numbers, since it would prolong the apparent “lag” observed before \([\psi^-]\) cells segregate. However, once \([\psi^-]\) cells start segregating, \(i.e.,\) when the numbers of propagons in cells are very low, the numbers do halve at each generation, because their appearance follows the appropriate halving kinetic. GdnHCl must therefore take its effect immediately, since the exponential decline in numbers extrapolated back to the time of addition of GdnHCl.

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**Figure 7.—Evidence for a mother bias in transmission of \([\psi^+]\) propagons in GdnHCl-grown cells.** (A) A scatter plot of the numbers of \([\psi^+]\) cells found in colonies growing on \(\frac{1}{2}YE + 3\,\text{mm}\,\text{GdnHCl}\). The colonies were grown from micromanipulated individual three-cell clusters taken from a logarithmic culture of 74-D694 \([\psi^+]\) (strong) growing in YPD + 3 mm GdnHCl liquid medium. The clusters were separated into “mother” (budded) and “daughter” (unbudded) cells. Clusters were sampled at 4 (○) or 6 (△) hr after addition of GdnHCl to the growing culture. The number of \([\psi^+]\) cells in each mother colony is plotted against the number in the corresponding daughter colony. (B) As in A except the three-cell clusters were taken from a culture of 74-D694 \([\psi^+]\) (strong) grown in YPD. Note that on both plots, a line with a slope of 1 has been drawn, \(i.e.,\) 50:50 mother:daughter distribution.
TABLE 3

The phenotypes of colonies derived from mother and daughter cells growing on \( \frac{1}{4} \)YE after several generations of growth in YPD + 5 mM GdnHCl medium

<table>
<thead>
<tr>
<th>Mother:</th>
<th>W</th>
<th>R</th>
<th>W</th>
<th>R/W</th>
<th>R</th>
<th>W</th>
<th>R/W</th>
<th>No. of ([PSI^+]/H11001) propagons/cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daughter:</td>
<td>W</td>
<td>R</td>
<td>( \bar{R} )</td>
<td>( \bar{R} )</td>
<td>( \bar{R} )</td>
<td>( \bar{R} )</td>
<td>( \bar{R} )</td>
<td>( \bar{R} )</td>
</tr>
<tr>
<td>BSC783/4a</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0</td>
<td>4</td>
<td>5</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>20</td>
<td>5</td>
<td>23</td>
<td>0</td>
<td>3</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>20</td>
<td>10</td>
<td>( \bar{M} )</td>
<td>( \bar{M} )</td>
<td>( \bar{M} )</td>
<td>( \bar{M} )</td>
<td>( \bar{M} )</td>
<td>( \bar{M} )</td>
</tr>
<tr>
<td>74-D694</td>
<td>1</td>
<td>5</td>
<td>5</td>
<td>4</td>
<td>3</td>
<td>1</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1</td>
<td>18</td>
<td>5</td>
<td>7</td>
<td>( \bar{T} )</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>6</td>
<td>23</td>
<td>( \bar{T} )</td>
<td>( \bar{T} )</td>
<td>( \bar{T} )</td>
<td>( \bar{T} )</td>
<td>( \bar{T} )</td>
<td>( \bar{T} )</td>
</tr>
</tbody>
</table>

W, \([PSI^+]/H11001\]; R, \([psi^-]/H11002\). Columns showing mother:daughter pairs where segregation of \([PSI^+]/H11001\) and \([psi^-]/H11002\) occurred are underlined. Columns 5 and 6 are where \([PSI^+]/H11001\) propagons have remained in the mother, and column 7 is where they have segregated to the daughter. The final column shows the number of propagons per cell estimated from the frequency of \([psi^-]/H11002\) colonies found in each sample.

Nonrandom segregation of propagons: Our data are mostly consistent with the random segregation model of Eaglestone et al. (2000). However, direct assays of the segregation of propagons into mother and daughter cells showed a consistent bias toward retention of propagons in the mother cell both when there were large numbers of propagons in the cells, i.e., in the early stages of curing, and when there were only one or two propagons per cell and \([psi^-]/H11002\) cells were segregating at high frequency. This has important implications for both the kinetics of curing and our understanding of the propagation of the \([PSI^+]/H11001\) prion determinant (Cole 2003).

The bias toward retention of propagons in the mother cell was particularly strong when the numbers of propagons in the cells were predicted to be low (Table 3). Although GdnHCl inhibits propagon generation, it does not inhibit the accretion of newly synthesized Sup35p to aggregates with the rate of accretion being proportional to the numbers of propagons (Ness et al. 2002). Therefore, in the presence of GdnHCl, the Sup35p aggregates might become very large and would be difficult to transmit to a daughter cell. Indeed, using a Sup35pNM-GFP fusion, Wegrzyn et al. (2001) observed an apparent increase in the size of prion aggregates in the presence of GdnHCl. If such aggregates are formed on (or by) propagons and physically include them, then an increasing bias in segregation might develop as growth in GdnHCl continued. This would have an effect on the estimates of propagon numbers we make by either method of analysis, leading to an earlier appearance of \([psi^-]/H11002\) cells in the culture and also preventing complete segregation of all propagons initially present in a cell into separate cells by the end of colony growth.

The prediction that \( n \) propagons will eventually segregate into \( n \) cells during growth in the presence of GdnHCl is supported by the recovery of the expected numbers of \([PSI^+]/H11001\) cells from colonies after sufficient growth. These data are also consistent with our previous assertion that propagons are not destroyed to any significant extent in the presence of 3 mM GdnHCl (Ness et al. 2002). The ranges of numbers of propagons found in populations of cells, by estimations from the kinetics of GdnHCl-induced curing, are matched by the ranges of numbers found in individual cells and colony-forming units (CFUs).

Propagon number and stable prion propagation: Our findings have important implications for understanding the nature of the prion phenomenon in yeast. Our data suggest that the numbers of \([PSI^+]/H11001\) propagons, defined as those versions of the prion protein—be they monomeric or multimeric—which replicate or increase in number as opposed to size—are very low. The number of Sup35p molecules in a cell is of the order of 10⁴, based on the number of Sup35p molecules relative to the more abundant ribosomal proteins (1:10; Didenchenko et al. 1991). If prion formation were merely an autocatalyzed cycle of template aggregation and dissociation, this would rapidly and (exponentially) generate comparable numbers of propagons, which would be too high a number to be consistent with our observations.

One possible scenario is that a propagon is an aggregate of prions. In one model, there are two processes: generation and accretion. The latter would perhaps require the ends of amyloid fibers formed by aggregation and the former would consist of splitting the aggregates to form more ends and would be the process inhibited by GdnHCl (Wegrzyn et al. 2001; Ness et al. 2002). This would be an exponential process and ultimately predicts similarly overwhelming numbers of propagons, unless
it is exactly balanced by the exponential increase in cell numbers during log phase.

The key to the mitotic (and possibly meiotic) stability of the [\(PSI^+\)] determinant must lie in how the generation of propagons and of cells is coordinated. We provide evidence here that one component of such an integrated system might be the restriction of prion generation to S phase in growing cells. Another comes from the observation that, apparently, the doubling time of prion generation is \(\sim 20\) min (Ness et al. 2002), a time that allows for a threefold increase during S phase. In fact, the observed S-phase increase in a normal cell culture was a doubling, together with an increase in of propagons in a culture, but this is a question that remains to be addressed. This may have a genetic basis, but sometimes the exceptional numbers occur in strains isogenic with cultures that seem to be in the 50–100 range for prion numbers. There may be preculture conditions, such as long storage in stationary phase, that affect the average number of propagons in a culture, but this is a question that remains to be addressed.

The third important observation is the bias toward propagons remaining in mother cells. If this affected all cell divisions, then a population would rapidly accumulate a number of cells with such low numbers of propagons that they would frequently segregate [\(psi^-\)] cells. The bias may reflect the difference in size between the mother and daughter cells at the point of cell separation. The volume ratio of mother to daughter cells at the time of cytokinesis is generally \(\sim 3:1\), but can vary between 1:1 and 4:1; usually it is greater than the propagon number ratio of 2:1. It is clearly difficult to say whether partition is a passive process, but the great stability of [\(PSI^+\)] suggests an active partition or replication mechanism that prevents the numbers from falling too low.

Strain-independent variation in propagon numbers: Our prediction about the distribution of [\(PSI^+\)] propagons in a growing culture may account for many of our observations. For example, the uniformity of distribution of numbers of propagons in G\(_1\) and S-phase cells, compared with the high variance found in G\(_2\) and three-cell clusters, may reflect the fact that all single cells or small-budded cells picked from cultures are most probably daughters whereas large-budded cells and three-cell clusters may be found with cells of any age. In any sample of the latter types, one or two old mothers are likely to have accumulated large numbers of propagons. Old mothers may therefore be the source of the propagon jackpots we have observed.

The source of the variation in propagon numbers between cultures is intriguing. The kinetics of curing observed during growth in GdnHCl is determined by the average number of propagons in CFUs. This is likely to be the numbers in daughter cells and young mothers that together comprise up to 80% of growing populations. We have suggested that this number is likely to be \(\sim 60\) and, allowing for an average CFU of 2.5 cells, this should consistently give numbers of \(\sim 120–150\). These are the numbers we have observed in the strain 74-694D over several independent experiments, but were not so consistently observed when the experiments were done with the strain BSC783/4a (B. S. Cox and F. Ness, unpublished data).

The occasional occurrence of cultures with very high numbers of propagons (500–3000 per cell) remains unexplained and our studies do not provide a rational explanation to account for them. The observation may have a genetic basis, but sometimes the exceptional numbers occur in strains isogenic with cultures that seem to be in the 50–100 range for prion numbers. There may be preculture conditions, such as long storage in stationary phase, that affect the average number of propagons in a culture, but this is a question that remains to be addressed.

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