Gene Trap Tagging of *PROLIFERA*, an Essential *MCM2-3-5*–Like Gene in *Arabidopsis*

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Gene trap transposon mutagenesis can identify essential genes whose functions in later development are obscured by an early lethal phenotype. In higher plants, many genes are required for haploid gametophyte viability, so that the phenotypic effects of their disruption cannot be readily observed in the diploid plant body. The *PROLIFERA* (*PRL*) gene, identified by gene trap transposon mutagenesis in *Arabidopsis*, is required for megagametophyte and embryo development. Reporter gene expression patterns revealed that *PRL* was expressed in dividing cells throughout the plant. *PRL* is related to the *MCM2-3-5* family of yeast genes that are required for the initiation of DNA replication.

The plant life cycle alternates between sporophytic (diploid) and gametophytic (haploid) generations. In flowering plants, the haploid phase of the life cycle is reduced to just a few cell divisions, but the requirement for the haploid gametophyte to survive means that mutations in essential genes, such as cell division cycle genes, will not be transmitted by either the male or the female gametophyte or both (*1*). The functions of these genes in the diploid sporophyte (the plant body) are thus difficult to discern. Mutations affecting meiosis and gametophyte development have been described in maize (*2*) and *Arabidopsis* (*3*), but cell cycle mutations have not been described molecularly in plants as they have in animals (*4*). Domains of mitotic activity in plants have been defined in meristems and lateral organ primordia by labeling studies (*5*) and by in situ hybridization with cell division cycle gene homologs (*6*). The peripheral zone of the shoot apical meristem and the proximal domain of immature leaves are examples of zones with high mitotic activity. As in *Drosophila* (*7*), the phase of the cell cycle in plants may restrict developmental potential in some patterning processes, such as stomatal complex differentiation in the epidermis (*8*).

Insertional mutagenesis through use of gene traps and enhancer traps can be used to identify late as well as early functions of genes that are disrupted by insertion. This is because gene expression can be examined in viable heterozygotes even when loss-of-function mutations are lethal in the homozygous or haploid state (*9*, *10*). In plants, transfer DNA (T-DNA) vectors have been used to integrate promoter trap and enhancer trap reporter genes into the genome of higher plants by *Agrobacterium*-mediated transformation (*11*). However, T-DNA insertions cannot be remobilized and frequently comprise multiple copies at a single locus, thereby complicating interpretation of expression patterns. As an alternative, we have developed a gene trap transposon tagging system in the higher plant *Arabidopsis thaliana*, using the maize transposable elements *Activator* (*Ac*) and *Dissociation* (*Ds*), which also function in *Arabidopsis* (*12*, *13*). Gene trap reporter genes are preceded by splice acceptors so that insertion into introns results in reporter gene fusions (*10*, *14*). In our system, each *Ds* gene trap (*Ds*G) element carries a β-*glucuronidase* (*gusA* or *uidA*) reporter gene, preceded by splicing signals, which functions as an "exon trap" (*13*).

In a screen for gene trap expression in seedlings, we have identified a gene required for both gametophytic and sporophytic development. F_3 seedlings from 77 independent gene trap insertion lines were stained for GUS activity, and 18 lines were identified that exhibited GUS expression in some region of the seedling. GUS expression patterns in these lines varied from uniform expression throughout the seedling to expression localized to specific regions or tissues (*13*). In one line, GT148, GUS was expressed throughout the early leaf primordia (Fig. 1, A and B), but was eventually lost from the distal domain of immature leaves, where mitotic activity is first diminished (*15*). No GUS activity was observed in mature leaves. Staining was observed in root tips (*16*) and lateral root primordia (Fig. 1C), but not in differentiated root tissue. GUS was also expressed throughout young flower buds (Fig. 1D), but became localized to the carpels, and finally to the ovules (Fig. 1E) in older flowers. After fertilization, GUS was expressed uniformly in the embryo (Fig. 1F). Because the gene controlling GUS expression in line GT148 appears to be expressed primarily in proliferating cells in each tissue, the gene was named *PROLIFERA* (*PRL*).

Exon sequences upstream of the *Ds*G insertion in *PROLIFERA* were isolated through the use of 5′ RACE-PCR (*17*, *18*), and sequencing revealed that the *gusA* reporter had been fused in frame with an upstream sequence at the second splice acceptor (Fig. 2A). The RACE-PCR product was used to screen a flower complementary

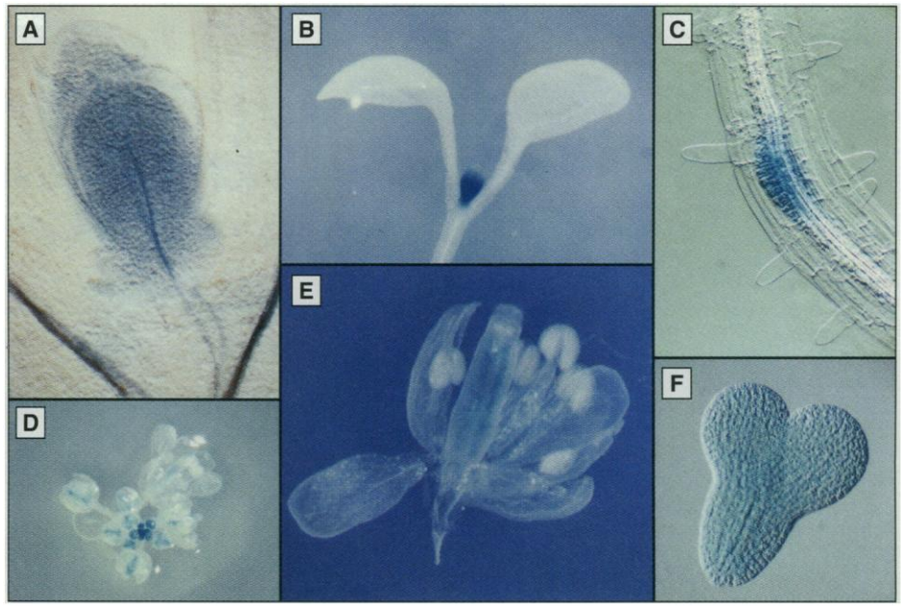

Fig. 1. Histochemical localization of GUS activity in gene trap line GT148. (**A**) Emerging primary leaves, stipules, and developing leaf primordia (Nomarski optics). (**B**) Immature leaf. (**C**) Lateral root primordia (Nomarski optics). (**D**) The inflorescence. (**E**) Ovules. (**F**) Heart stage embryo (Nomarski optics). Tissues were stained in X-Gluc (*30*).

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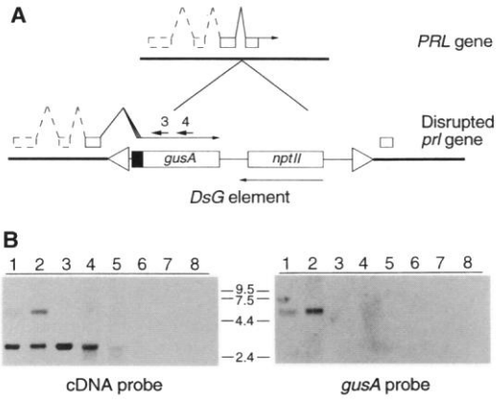
DNA (cDNA) library, and one of the cDNA clones was used to probe a Northern (RNA) blot from wild-type plants and plants heterozygous for the *prl::DsG* insertion (Fig. 2B). A 2.7-kb transcript was found in RNA from flowers and roots of heterozygous plants and in RNA from buds, flowers, and roots of wild-type *Arabidopsis*. No transcript was detected in RNA from mature leaves. An additional, 5.2-kb *PRL* transcript in RNA from heterozygous plants also hybridized with a probe specific for *gusA*, indicating that it was derived from a transcriptional fusion with the gene trap reporter gene (Fig. 2B). Thus, the GUS expression pattern appears to accurately reflect the expression pattern of the *PROLIFERA* gene.

The largest cDNA clone was 1.8 kb in size. RACE-PCR was used to isolate the remaining 5′ sequences (*19*). A single open reading frame of 716 amino acids was predicted from the cDNA sequence (*20*), and database searches (*20*) revealed strong homology with a family of proteins involved in DNA replication and cell division that includes MCM2, MCM3, and CDC46/MCM5 in *Saccharomyces cerevisiae*, cdc21$^+$ in *Schizosaccharomyces pombe*, and the mammalian P1 and BM28 proteins (*21*, *22*). A central 200–amino acid region is highly conserved in all members of this family, and the *PROLIFERA* sequence is 40 to 70% identical to the other proteins within this region. This region contains a conserved adenosine triphosphate–binding domain (*22*). PRL is most similar in sequence to the hypothetical yeast protein YBR1441 (*23*) identified by yeast genomic sequencing (41% identical overall and 71% identical in the conserved region) (Fig. 2C). Mutations in many of these genes, including YBR1441 (*24*), cause cell cycle arrest at the G_1 to S transition or in S phase.

On the basis of this homology, disruption of *PRL* might be expected to cause a visible phenotype, and plants heterozygous for the insertion were found to be semi-sterile (Fig. 3A). Each *Arabidopsis* ovule supports a single haploid megagametophyte (the embryo sac), which is derived from a haploid product of meiosis by three rounds of nuclear division. Semi-sterile *prl*/+ siliques were found to have some mutant embryo sacs arrested at various stages, suggesting that the *prl::DsG* insertion resulted in reduced megagametophyte viability (*25*). To confirm this, we carried out reciprocal crosses to wild-type plants to examine transmission of the *nptII* gene, carried by the *Ds*G element. Relative to the wild-type locus (100%), 95% transmission of kanamycin resistance was observed through the male and 50% through the female (Table 1), indicating that the mutation resulted in reduced megagametophyte viability. All kanamycin-resistant plants (n = 93), including those derived from *prl* female parents, were semi-sterile, indicating that the muta-



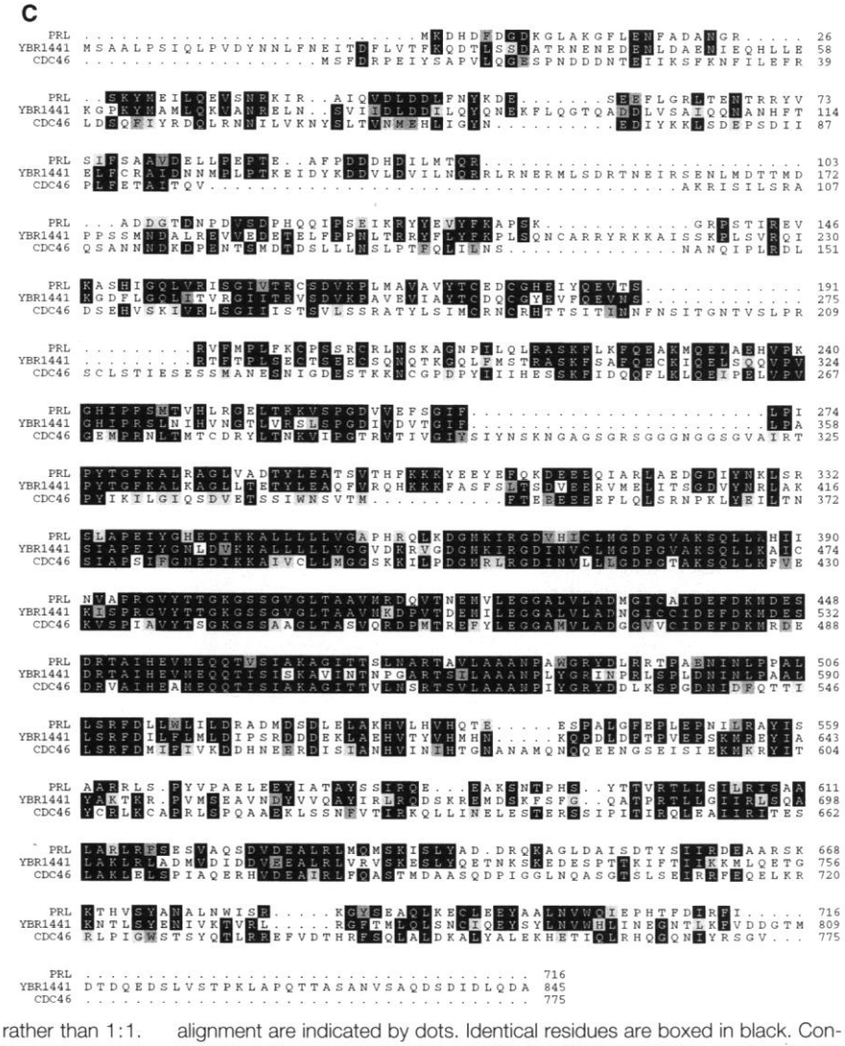


Fig. 2. (**A**) Structure of the *DsG* insertion in *PRL*. Boxes represent exons. The *DsG* element is inserted at nucleotide (nt) 62 of an 85-bp intron. The *PRL* gene contains at least two additional introns, although their exact size and location is not known (designated by dashed lines). Three splice acceptors are at positions 317, 328, and 339 from the end of the *DsG* element and are preceded by an intron from the *Arabidopsis GPA1* gene (*31*). The *gusA* gene begins 343 nt from the end of *DsG*. Nested primers 3 and 4 (*18*), used for RACE-PCR, are at positions 100 to 123 and 149 to 176, respectively, from the *gusA* ATG. *gusA* is β-*glucuronidase*, and *nptII* is *neomycin phosphotransferase*, which confers resistance to kanamycin. (**B**) RNA blot analysis. Approximately 10 μg of total RNA (*32*) isolated from roots (lane 1) and inflorescences (lane 2) of *prl*/+ heterozygotes, and from roots (lane 3), floral buds (lane 4), open flowers (lane 5), cauline leaves (lane 6), rosette leaves (lane 7), and stems (lane 8) of wild-type *Arabidopsis*, ecotype Landsberg *erecta*. The left panel shows transcripts detected by a *PRL* cDNA probe. The right panel shows transcripts detected with a *gusA* probe. The smaller transcript in open flowers detected by *gusA* probably represents artefactual hybridization to ribosomal RNA. Roots were collected from a segregating population of wild-type and heterozygous plants, so the ratio of the 5.2-kb transcript to the 2.7-kb transcript is 1:3 rather than 1:1. Molecular sizes are indicated in kilobase pairs. (**C**) Comparison of the amino acid sequences of the PRL, YBR1441, and CDC46 proteins (*33*). Gaps in the alignment are indicated by dots. Identical residues are boxed in black. Conserved residues are boxed in gray. The GenBank accession number for the PRL cDNA sequence is L39954.

tion was fully penetrant but leaky. No homozygous F_3 plants were found in 121 kanamycin-resistant plants examined. However, each heterozygous silique contained a few aborted seed (Fig. 3, C and D) that stained for GUS activity (*16*). Thus, transmission of the semi-sterility mutation through the female gametophyte led to variable lethality in homozygous embryos.

To demonstrate that the *Ds*G insertion was responsible for semi-sterility, we generated revertants by *Ds*G excision. *Ac*/− prl/+ F_1 plants were semi-sterile, but had occasional fully fertile siliques, indicating that the phenotype was unstable in the presence of *Ac* (*16*). One plant contained a large sector encompassing most of the primary bolt. Southern (DNA) analysis revealed that the *Ds*G element had excised in fully fertile plants derived from the revertant siliques in this sector, whereas heterozygous siblings derived from semi-sterile siliques retained the *Ds*G element (Fig. 4A). However, 8 out of 62 plants derived from revertant siliques were semi-sterile, indicating that the revertant sector did not encompass the entire flower (*26*). Genomic sequences were amplified from wild-type, *prl*/+, and revertant plants (*27*), and sequencing revealed that the *Ds*G element was inserted in an 85–base pair (bp) intron and created an 8-bp target site duplication (*12*). A 6-bp footprint remained after excision of the element (Fig. 4B).

Gametophyte lethality is leaky in *prolifera*. This could be because the element is inserted in an intron close to the 3′ end of the gene, so that the truncated fusion protein retains some activity. Alternatively, there may be related genes that can partially substitute for *PRL*, particularly in the male gametophyte, which is unaffected by the mutation. Additional genomic DNA fragments were detected by low-stringency hybridization, supporting this idea (*16*). In contrast, a similar mutation in *Arabidopsis*, *gametophyte factor1* (*3*), causes female gametophyte lethality and also affects transmission through the male.

MCM2-3-5 proteins are nuclear-localized in yeast and are required for the initiation of DNA replication from particular replication origins. They have been proposed to act as the replication licensing factor (*22*), which regulates initiation of DNA replication during the cell cycle (*28*). Consistent with a role in DNA replication, *PRL* is required very early, during megagametophyte development, but also has a more general role in dividing cells throughout the plant that could only have been identified through the use of gene traps. The staining pattern we observed could be used to screen for other genes involved in cell division and may aid in the analysis of morphogenetic mutants by defining zones of active DNA replication.

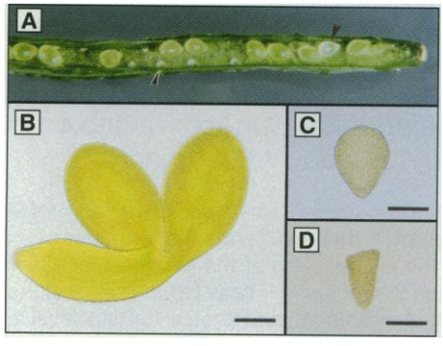


Fig. 3. Semi-sterile phenotype in *prl* heterozygotes. (**A**) Maturing silique dissected to expose developing seeds, unfertilized ovules (white arrowhead), and seed containing aborted embryos (black arrowhead). Wild-type embryo (**B**) and defective embryos (**C** and **D**) from such a silique. The size bars in (B) through (D) represent 0.1 mm.

Table 1. *DsG* transmission in *prl*/+ heterozygotes. Outcrosses with *prl*/+ heterozygotes either as males or females to wild-type plants were performed, and the *prl*/+ parents were also selfed. The *DsG* element carries the *nptII* gene, so that kanamycin resistance was used to follow the *DsG* element. Data from 40 crosses were pooled with both Landsberg and Columbia ecotypes as the wild-type parent. Reduced female transmission was significant at $P < 0.01\%$. All Kan-r progeny tested were also semi-sterile.

Female	Male	Kan-r	Kan-s
prl/+	+/+	205	407
+/+	*prl*/+	425	445
prl/+	selfed	2705	2010

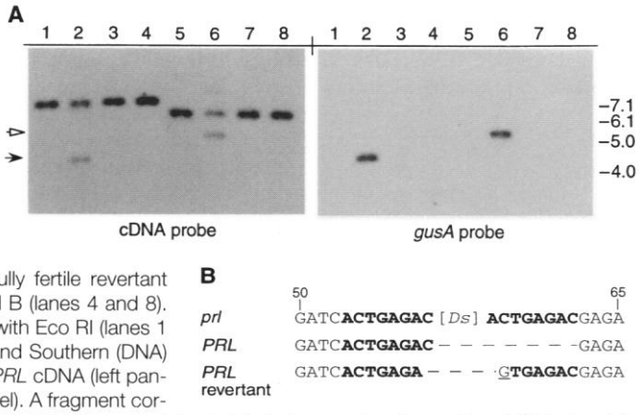


Fig. 4. Phenotypic reversion of semi-sterility by *DsG* element excision. Semi-sterile and fully fertile siliques were selected from a *prl*/+; *Ac*/− plant. (**A**) DNA gel blot analysis of wild-type Landsberg *erecta* (lanes 1 and 5); *prl*/+, without *Ac* (*29*) (lanes 2 and 6); and pooled seedlings from fully fertile revertant siliques A (lanes 3 and 7) and B (lanes 4 and 8). Genomic DNA was digested with Eco RI (lanes 1 to 4) or Xba I (lanes 5 to 8), and Southern (DNA) analysis was performed with *PRL* cDNA (left panel) and *gusA* probes (right panel). A fragment corresponding to the *DsG* element insertion was detected in heterozygotes (lanes 2 and 6), but not in wild-type (lanes 1 and 5) or revertant (lanes 3, 4, 7, and 8) plants (arrows). No *DsG* fragments were detected in DNA from revertant plants, indicating that the *DsG* element excised, and did not reinsert. Molecular size markers are in kilobase pairs. (**B**) DNA sequences flanking the *DsG* element insertion in *prl*. The 8-bp sequence in boldface is the target site duplication. Numbers refer to nucleotide positions in the intron, which lies between amino acids 684 and 685 in the open reading frame shown in Fig. 2C. A 6-bp footprint remains after *DsG* excision. The underlined G is not derived from the target site duplication.

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13. The gene trap and enhancer trap transposon tagging system we have developed is described in detail elsewhere (V. Sundaresan *et al.*, unpublished results). In brief, transgenic plants carrying a single T-DNA insert containing both the *DsG* element and a constitutive *indole–acetic acid hydrolase* gene, *iaaH*, driven by the 2′ T-DNA promoter [I. Bancroft *et al.*, *Mol. Gen. Genet.* **233**, 449 (1992)], were crossed to transgenic plants carrying a single T-DNA insert containing a 35*S* promoter::*Ac* transposase gene [S. R. Scofield, J. J. English, J. D. G. Jones, *Cell* **75**, 507 (1993)] and another *iaaH* gene. The resulting F_1 plants were selfed, and lines carrying transposed *DsG* elements were selected among the F_2 progeny seed by sowing them on agar plates containing α-naphthalene acetamide (NAM) and kanamycin. Seedlings carrying either T-DNA insert were stunted because of the *iaaH* gene that conferred sensitivity to NAM [G. A. Karlin-Neumann, J. A. Brusslan, E. M. Tobin, *Plant Cell* **3**, 573 (1991)]. Of the remaining seedlings, only those carrying a transposed *DsG* element survived on kanamycin, by virtue of a 1′ T-DNA promoter::*nptII* gene fusion [J. D. G. Jones

et al., *Transgenic Res.* **1**, 285 (1992)] carried within the *DsG* element. In addition, the *DsG* element contained a *gusA* reporter gene in opposite orientation to the *nptII* gene (Fig. 2A). Note that the *nptII* gene is contained within the *Ds* element, not within the flanking T-DNA. Seedlings carrying independent transposed elements were transplanted to soil and allowed to self-fertilize, giving rise to a gene trap line. The seeds from each line were sown on agar and stained for GUS activity 3 to 7 days after germination.
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18. Complementary DNA synthesis was carried out with 10 μg of total RNA isolated from flowers of *prl* heterozygous plants and GUS4 primer (5′-GCTCTAGATCGGCGAACTGATCGTTAAAAC-3′). Second-strand synthesis and polymerase chain reaction (PCR) were carried out with oligo(dT)$_{17}$-adaptor and Adaptor primers (*17*) and GUS3 primer (5′-GAGAATTCTTGTAACGCGCTTTCCCACCA-3′). PCR reactions were heated to 95°C for 5 min, followed by 40 cycles of amplification (95°C, 1 min; 53°C, 1 min; 72°C, 1.5 min) and 10-min final extension at 72°C. Products were size-fractionated, reamplified with Adaptor and GUS3 primers, gel-purified, and cloned into M13.
19. Complementary DNA synthesis was carried out with 5 μg of total RNA isolated from floral buds of wild-type plants and primer 148C (5′-GGGAGCTCTTGCATCTTGGCCTCCTGAAAC-3′). Second-strand cDNA synthesis and PCR were done with the Q_T and Q_O primers [M. A. Frohman, *Methods Enzymol.* **218**, 340 (1993)] and primer 148B (5′-GGGAATTCCCGGCTTTACTATTAAGACG-3′). Reamplification was done with Q_I and 148B primers. Amplified products were gel-purified after size selection and cloned into pBluescript.
20. Double-stranded template DNA was isolated with the use of Qiawell strips (Qiagen, Inc., Chatsworth, CA) and previously described modifications of the Qiagen protocol [W. R. McCombie, C. Heiner, J. M. Kelley, M. G. Fitzgerald, J. D. Gocayne, *DNA Sequencing* **2**, 289 (1992)]. Cycle sequencing reactions were carried out with fluorescent forward and reverse primers (Applied Biosystems, Foster City, CA) and SequiTherm polymerase (Epicenter Technology, Madison, WI). The sequences of overlapping clones were assembled with the use of Xbap software [T. J. Gleeson and R. Staden, *Comput. Appl. Biosci.* **7**, 398 (1991)]. Gaps remaining after assembly of the subcloned sequences were filled with custom primers and either Sequenase dye terminator or Taq dye terminator kits (Applied Biosystems). All sequence data were acquired through use of ABI373 DNA sequencers with 34-cm stretch liners (Applied Biosystems). Databases were searched with the Blast program [S. F. Altschul, G. Warren, W. Gish, E. W. Myers, D. J. Lipman, *J. Mol. Biol.* **215**, 403 (1990)].
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Role of the Chaperone Protein Hsp104 in Propagation of the Yeast Prion-Like Factor [*psi*$^+$]

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The yeast non-Mendelian factor [*psi*$^+$] has been suggested to be a self-modified protein analogous to mammalian prions. Here it is reported that an intermediate amount of the chaperone protein Hsp104 was required for the propagation of the [*psi*$^+$] factor. Overproduction or inactivation of Hsp104 caused the loss of [*psi*$^+$]. These results suggest that chaperone proteins play a role in prion-like phenomena, and that a certain level of chaperone expression can cure cells of prions without affecting viability. This may lead to antiprion treatments that involve the alteration of chaperone amounts or activity.

Certain mammalian neurodegenerative diseases, such as sheep scrapie, human Creutzfeldt-Jacob disease, and bovine spongiform encephalopathy, are widely believed to be caused by proteins in an unusual protease-resistant conformation, called prions (*1*). Evidence indicates that transmission of the prion diseases does not require any nucleic acid. Rather, infection depends on the prion's ability to convert unmodified nonprion protein, encoded by the same host gene, into the prion conformation. It has been noted that the yeast factors [*URE3*] (*2*) and [*psi*$^+$] (*2*, *3*) closely resemble prions.

The [*psi*$^+$] factor was first described as a non-Mendelian element, found in some but not other laboratory strains of *Saccharomyces cerevisiae*, that increases the efficiency of certain nonsense suppressor transfer RNAs [for a review, see (*4*)]. Later it was shown that [*psi*$^+$] causes weak nonsense suppression by itself (*4*–*6*). No extrachromosomal DNA or RNA elements have been found to be responsible for [*psi*$^+$] (*4*). Moreover, [*psi*$^+$] can be "cured" (lost from the cell) by stress-inducing agents (*4*). Several pieces of evidence strongly suggest (*2*, *3*) that [*psi*$^+$] is a prion-like form of the EF-1α–related protein Sup35. (i) Mutations in the *SUP35* gene cause omnipotent suppression, just as [*psi*$^+$] does (*7*). (ii) Overexpression of *SUP35* induces [*psi*$^+$] (*8*), just as overexpression of the prion protein gene (*PrP*) in transgenic mice induces the prion disease

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