Spo12 Is a Limiting Factor That Interacts With the Cell Cycle Protein Kinases Dbf2 and Dbf20, Which Are Involved in Mitotic Chromatid Disjunction

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ABSTRACT

The *DBF2* and *DBF20* genes of the budding yeast *Saccharomyces cerevisiae* encode a pair of structurally similar protein kinases. Although yeast with either gene deleted is viable, deletion of both genes is lethal. Thus, the Dbf2 and Dbf20 proteins are functional alternatives for an essential activity. In contrast to deletions, four different mutant alleles of *DBF2* are lethal. Thus, the presence of a nonfunctional Dbf2 protein, rather than the lack of function *per se*, is inhibitory. Here we present genetic evidence that nonfunctional mutant Dbf2 protein blocks the function of Dbf20 protein by sequestering a common interacting protein encoded by *SPO12*. Even a single extra copy of *SPO12* is sufficient to suppress the *dbf2* defect. Since *SPO12* appears to encode a limiting factor, it may be a rate limiting cofactor that is involved in the regulation of the Dbf2 and Dbf20 protein kinases. A corollary to the finding that one extra copy of *SPO12* can suppress *dbf2*, is that the acquisition of an extra chromosome *VIII*, which carries the *SPO12* locus, will also suppress *dbf2*. Indeed, physical analysis of chromosome copy number in *dbf2* revertants able to grow at 37° showed that the frequency of chromosome *VIII* acquisition increased when cells were incubated at the restrictive temperature, and reached a frequency of more than 100-fold the amount in wild-type yeast. This suggested that the *dbf2* mutation was not only suppressed by an extra copy of chromosome *VIII* but also that the *dbf2* mutation actually caused aberrant chromosomal segregation. Conventional assays for chromosome loss confirmed this proposal.

In eucaryotic cells, many of the processes of the cell division cycle are regulated by phosphorylation of cellular proteins. A number of protein kinases and phosphatases involved in this phosphorylation have been described (HOEKSTRA, DEMAGGIO and DHILLON 1991a, 1991b; SUTTON, IMMANUEL and ARNDT 1991; MILLAR and RUSSELL 1992). The kinases and phosphatases are themselves regulated, and one of the mechanisms by which this is achieved involves complex formation with other proteins, which act as regulatory subunits. Examples of regulation by complex formation are the $p34^{cdc2}$ kinase, which complexes with cyclins (REED 1991), the cAMP-dependent kinases, which consist of regulatory and catalytic subunits (KREBS and BEAVO 1979) and the Cdc7 cell cycle kinase, which complexes with the protein encoded by *DBF4* (KITADA *et al.* 1992; JACKSON *et al.* 1993).

Another protein kinase, which is encoded by the *DBF2* gene in budding yeast, is required at a late stage in the cell cycle for completion of mitotic division. At the restrictive temperature for growth, *dbf2 ts* mutants arrest at a late stage in nuclear division with a uniform large-budded terminal phenotype, which we call "*d*umb*b*ell *f*ormation." As well as causing a late cell cycle phenotype, *dbf2* causes a delay in the onset of DNA replication during S phase, suggesting that the Dbf2 protein also acts at a stage early in the cell cycle (JOHNSTON *et al.* 1990). Although the existing *dbf2* mutations are recessive and lethal, deletion of the *DBF2* gene does not affect cell viability. Survival of cells deleted for *DBF2* depends on a second gene, *DBF20*, which also encodes a protein kinase, and is homologous to *DBF2*. Apparently, the recessive alleles of *dbf2* are able to exclude *DBF20* from substituting for *dbf2* at the restrictive temperature (TOYN *et al.* 1991).

In this paper we present genetic evidence that the Dbf2 protein excludes Dbf20 by sequestering a protein cofactor that is present in a limited amount, and that the cofactor is the product of the *SPO12* gene (MALAVASIC and ELDER 1990). *SPO12* was originally identified from a null mutation causing a defect in sporulation, in which meiosis I is bypassed, resulting in asci that contain two diploid spores (KLAPHOLZ and ESPOSITO 1980). *SPO12* was subsequently found in a separate screen for genes able to suppress a *dbf2* mutation, suggesting that it also has a mitotic role. Consistent with a mitotic role for *SPO12*, expression of *SPO12* mRNA, like *DBF2*, is under cell cycle control, the two of them being expressed simultaneously late in the mitotic cell cycle (PARKES and JOHNSTON 1992).

TABLE 1

Yeast strains

Name	Genotype	Source
J1	Heterozygous *dbf2* diploid	J114 × J189
J2	Homozygous *dbf2* diploid	Mitotic segregant of J1
J99	*MAT***a** *dbf20Δ::TRP1 trp1-289 ura3-52 leu2-3,112 ade5 can1*	TOYN *et al.* (1991)
J102-2D	*MATα dbf2-1 dbf20Δ::TRP1 trp1-289 ura3-52 ade1/5*	J99 × L119-7D
J102-3C	*MAT***a** *dbf2-1 dbf20Δ::TRP1 trp1-289 ura3-52 leu2-3,112 ade1/5*	J99 × L119-7D
J103-2A	*MATα dbf2-2 dbf20Δ::TRP1 trp1-289 ura3-52 leu2-3,112 ade1/5*	J99 × L181-6B
J103-6B	*MATα dbf2-2 dbf20Δ::TRP1 trp1-289 ura3-52 leu2-3,112*	J99 × L181-6B
J113	*MAT***a** *dbf2-2 dbf20Δ::TRP1 trp1-289 arg4*	This study
J114	*MAT***a** *dbf2-2 arg4 trp1-289*	This study
J145	*MATα dbf2-2 ura3-52 leu2 trp1 ade2-101*	YPH98 × J103-2A
J149	*MAT***a** *dbf2-2 ura3-52 leu2 trp1 ade2-101*	YPH98 × J103-2A
J157	*MATα dbf2-2 dbf20Δ::TRP1 trp1 ura3-52 leu2-3,112 ade2-101*	YPH98 × J103-2A
J159	*MAT***a** *dbf2Δ::LEU2 leu2 ura3-52 trp1 ade2-101*	This study
J189	*MATα ura3-52 his1 trp2 can1 ade5 leu2-3,112*	This study
J211-2C	*MAT***a** *dbf2Δ::LEU2 dbf20Δ::TRP1 leu2 trp1 ura3 ade2-101 + pASZ11-DBF2*	This study
J211-14C	*MATα dbf2Δ::LEU2 leu2 ura3 trp1 ade2-101*	This study
J226-1	*MAT***a** *dbf2Δ::LEU2 spo12Δ::TRP1 leu2 trp1 ura3 lys2 his3/7 ade2 + pASZ11-DBF2*	This study
J226-3	*MATα dbf2Δ::LEU2 spo12Δ::TRP1 leu2 trp1 ura3 lys2 his3/7 ade2 + pASZ11-DBF2*	This study
J226-5	*MATα dbf2Δ::LEU2 spo12Δ::TRP1 leu2 trp1 ura3 lys2 ade2 + pASZ11-DBF2*	This study
J226-6	*MATα dbf2Δ::LEU2 spo12Δ::TRP1 leu2 trp1 ura3 his3/7 ade2 + pASZ11-DBF2*	This study
L119-7D	*MATα dbf2-1 ura3-52 trp1-289 ade1*	PARKES and JOHNSTON (1992)
L181-6B	*MATα dbf2-2 ura3-52 leu2-3,112 trp1-289*	This study
CG378	*MAT***a** *ura3-52 trp1-289 leu2-3,112 ade5 can1*	C. GIROUX
S7-4A	*MAT***a** *dbf2Δ::URA3 ura3 leu2 trp1 ade5 his7*	TOYN *et al.* (1991)
S7-4B	*MATα dbf2Δ::URA3 ura3 leu2 trp1 ade5*	TOYN *et al.* (1991)
S2-2D	*MATα dbf2Δ::LEU2 leu2 ura3 trp1 ade5 can1*	TOYN *et al.* (1991)
YPH98	*MAT***a** *ura3-52 leu2-Δ1 trp1-Δ1 ade2-101 lys2-801*	SIKORSKI and HIETER (1989)
D273	*MATα ade1 his1 trp2*	JOHNSTON and THOMAS (1982)

A serendipitous finding was that an extra copy of chromosome *VIII*, and hence extra copy of *SPO12*, caused suppression of *dbf2*. This pointed to *dbf2* causing a high frequency of chromosome acquisition, presumably as a result of nondisjunction of sister chromatids during M phase. Since further experiments showed that *dbf2* also caused a high level of chromosome loss, this suggested that *DBF2* is important for sister chromatid separation, consistent with its role during nuclear division.

MATERIALS AND METHODS

Yeast strains and media: Table 1 lists the genotypes and sources of the strains used in this work. YPD medium was 1% Difco yeast extract, 2% Bacto peptone and 2% glucose. YPD plates were YPD containing 2% agar. Auxotrophy was scored on Wickerham's synthetic minimal medium with appropriate additions (WICKERHAM 1951). Incubation was at a "permissive temperature" of 25° or a "restrictive temperature" of 37°.

Plasmid DNA: For high copy number expression of *SPO12*, an 0.8-kb *Eco*RI genomic restriction fragment containing *SPO12* (MALAVASIC and ELDER 1990) was cloned into the *Pvu*II site of YEp24 (New England Biolabs, CP Laboratories, Bishop's Stortford, U.K.). For integration of a single copy of *SPO12*, the 0.8-kb fragment containing *SPO12* was cloned into the *Eco*RI site of pRS304 (SIKORSKI and HIETER 1989). For integration of a single copy of *DBF2*, a 4.2-kb genomic *Bam*HI fragment containing *DBF2* was cloned into the *Bam*HI site of pRS304. The resulting plasmids, pRS304-*SPO12* and pRS304-*DBF2*, as well as the parental plasmid pRS304, were linearized by cutting in the *TRP1* marker gene using the restriction enzyme *Sna*BI before integrative transformation at the *TRP1* locus of yeast. The plasmid pASZ11 was a gift from A. STOTZ (STOTZ and LINDER 1990). The plasmid pASZ11-*DBF2* had the 4.2-kb genomic *Bam*HI fragment containing *DBF2* cloned into the *Bam*HI site of pASZ11. The Thr^{195} allele of *DBF2* was expressed using the same 4.2-kb genomic *Bam*HI fragment cloned into the *Bam*HI site of YRp12 (JOHNSTON *et al.* 1990). Plasmid DNA was introduced into yeast by the method of ITO *et al.* (1983).

Analysis of suppression in *dbf2* mutants: Plasmids were introduced into yeast cells (ITO *et al.* 1983), and transformants were selected on minimal media at 25° for 3 or 4 days. Yeast colonies that appeared after this time were replica-plated to YPD plates that were then incubated overnight at 25° and 37°. Suppression was affirmed when all colonies were able to grow at 37°.

Pulsed field electrophoresis and preparation of chromosome-sized DNA: Preparation of chromosome-sized DNA was carried out by digestion of stationary phase yeast cells suspended in agarose blocks, as described by SCHWARTZ and CANTOR (1984). Yeast chromosomes were separated by CHEF (contour-clamped homogeneous electric field) gel electrophoresis (CHU, VOLLRATH and DAVIS 1986), using a Biorad CHEF-DR II megabase DNA pulsed field electrophoresis system (Biorad Laboratories Ltd. Hemel Hempstead, U.K.). Chromosomes *V* and *VIII* of strain J114 had very similar mobilities, but were resolved under the follow-

ing conditions. The gel was 1% agarose (SeaKem, cat. no. 50014, FMC BioProducts, Rockland, Maine 04841) and the running conditions were 170 V for 24 hr at 15°, with a switching time of 50 sec.

Southern hybridization: DNA was extracted from yeast by the method of HOFFMAN and WINSTON (1987). Genomic restriction fragments were transferred to membranes and hybridized under conditions of high stringency, as previously described (TOYN *et al.* 1991). The following hybridization probes were used; a 1.3-kb *Eco*RI fragment of *DBF2* (JOHNSTON *et al.* 1990), an 0.8-kb *Eco*RI fragment of *SPO12* (MALAVASIC and ELDER 1990), and a 1.3-kb *Bgl*II fragment of *DBF3* (J. SHEA, J. H. TOYN and L. H. JOHNSTON, unpublished data). Autoradiograms of the Southern blots were quantitated by scanning densitometry using a Chromoscan 3 machine (Joyce Loebl, Gateshead, Tyne and Wear, U.K.).

Source of the revertants that were used in the the pulsed-field electrophoresis experiment: Single cells from a log phase culture of the *dbf2* strain J114 were spread on YPD plates (10^5 per plate) and incubated at 37°. For half of the plates, after incubation for 5 hr at 37°, this was followed by a 1-hr "recovery" period at 25°, before further incubation for 3 days at 37°. The frequency of reversion among the cells that had been given the 1-hr "recovery" period at 25° was 1.1×10^{-3} per cell originally plated, compared with 1×10^{-4} for cells that had not had a "recovery" period. Thus, more than 90% of the revertants had arisen on the YPD plates during this experiment as a result of the cell cycle block followed by the "recovery" period, and were therefore not the result of any "jackpot" effect and hence not clonally related.

Chromosome *V* loss assay: Strains J1 and J2 were incubated at different temperatures, as described in the text. Subsequently, 10^5 cells were spread on a YPD plate and incubated at 25° overnight. During this incubation, small colonies of cells became just visible. These colonies were then replicated to plates containing 5-fluoro-orotic acid (BOEKE, LACROUTE and FINK 1984) to select for $Ura3^-$ clones. After incubation for 3 days at 25°, the colonies that appeared were replicated to test for histidine and uracil auxotrophies. Clones that were simultaneously Ura^- and His^- were taken to indicate loss of chromosome *V*. In all chromosome *V* loss assays, parallel samples of 100 or 200 cells were spread onto YPD plates, and the number of colonies formed after 3 days' incubation at 25° was recorded, in order to estimate the number of viable cells present at the time of sampling.

RESULTS

***dbf2* mutations block the function of wild-type *DBF20*:** The *dbf2* temperature-sensitive (*ts*) mutations are loss-of-function alleles and are conditionally lethal. In contrast, the *dbf2*Δ mutant is viable, although it is also a loss-of-function allele. The *dbf2*Δ strains are viable because of the *DBF20* gene, a close homologue of *DBF2*. *DBF20*, like *DBF2*, can be deleted from yeast without loss of viability. However, the double deletion, *dbf2*Δ *dbf20*Δ, is lethal (TOYN *et al.* 1991). Thus, the presence of a *dbf2 ts* allele appears to block the function of *DBF20*. One possible interpretation is that the mutant Dbf2 protein is sequestering a limiting factor into a nonproductive complex. A prediction based on this hypothesis is that a site-directed point mutation in *DBF2* resulting in loss of function, but not a gross alteration in protein structure, would be lethal in a *dbf2*Δ genetic background. To test this, we used a plasmid-borne clone of *DBF2* in which Lys^{195} had been mutated to threonine (see MATERIALS AND METHODS). Lys^{195} corresponds to the conserved lysine residue of catalytic subdomain II, found in all protein kinases, and is important for kinase activity but not for protein structure (HANKS, QUINN and HUNTER 1988). The Thr^{195} mutation did not rescue the *ts* defect of *dbf2* mutants (JOHNSTON *et al.* 1990), nor did it affect growth when expressed in wild-type yeast. However, despite the appearance of being nonfunctional, it proved impossible to introduce the Thr^{195} mutant into a *dbf2*Δ strain, even though a control plasmid in the same transformation mix was readily taken up (Table 2). Apparently, the nonfunctional *dbf2*-Thr^{195} allele behaved as if it were dominantly negative in the *dbf2*Δ background. Thus, the nonfunctional Dbf2 protein appears to sequester a protein that is present in a limited amount, and to which the wild-type version of Dbf2 would normally be bound, thereby preventing the access of Dbf20 to this limiting factor. This implies that the limiting factor has a higher affinity for Dbf2 than for Dbf20.

Spo12 has the genetic properties expected for the limiting factor: Two predictions from the "limiting factor" hypothesis are that overexpression of the limiting factor would, first, suppress all recessive *dbf2 ts* alleles, and second, only be able to do so in the presence of *DBF20*. A number of genes have been

TABLE 2

A nonfunctional point mutation in *DBF2* is lethal in a *dbf2*Δ background

Transformants	J159 (*dbf2*Δ)	YPH98 (*DBF2*)
Ura^+ (p*DBF2*-Thr^{195})	0	>1000
Ade^+ (pASZ11)	ca. 800	>1000
Ura^+Ade^+ (both plasmids)	0	ca. 1000

A mixture containing 0.5 μg each of plasmids p*DBF2*-Thr^{195} and pASZ11 was transformed into yeast strains J159 and YPH98. Transformation mixtures were split three ways and independent selection for Ura^+, Ade^+ and Ura^+Ade^+ was carried out. The numbers of transformants obtained are shown above.

TABLE 3

Suppression of *dbf2* by SPO12 requires *DBF20*

Strain	Genotype	Suppression
J102-2D	*dbf2-1 dbf20*Δ	No
J102-3C	*dbf2-1 dbf20*Δ	No
J157	*dbf2-2 dbf20*Δ	No
L119-7D	*dbf2-1 DBF20*	Yes
J145	*dbf2-2 DBF20*	Yes

A multicopy vector carrying the *SPO12* gene was introduced into the above strains and suppression was assessed as described in MATERIALS AND METHODS.

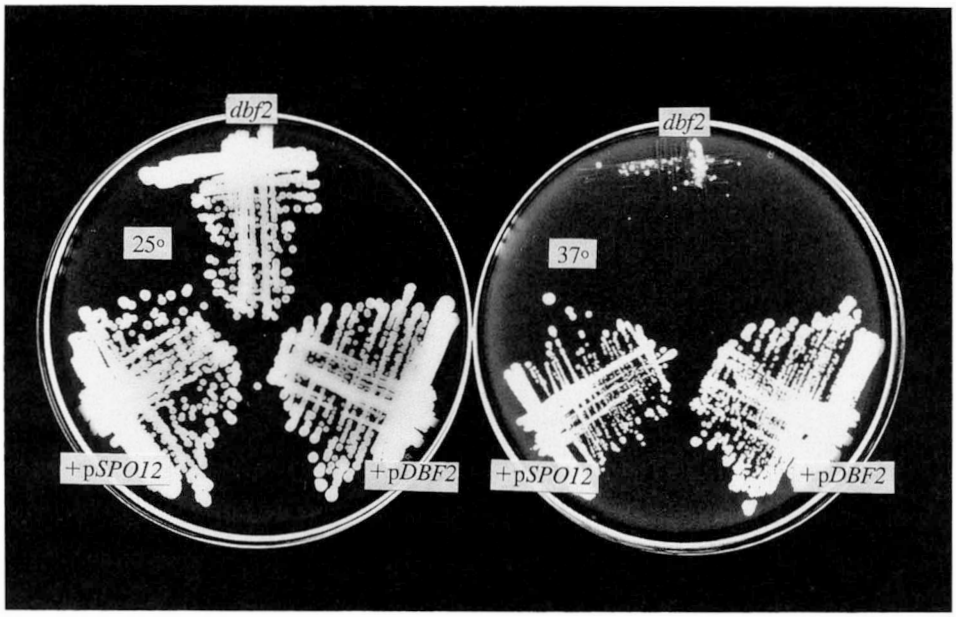

FIGURE 1.—A single extra copy of *SPO12* suppresses *dbf2*. A single copy of *SPO12* or *DBF2* was introduced into strain J114 using integrating plasmids. The *dbf2* control contained only the parental plasmid (see MATERIALS AND METHODS). A transformed clone of each was then streaked out onto YPD agar and incubated for 3 days at 25° or 37° as indicated.

cloned that are high copy number suppressors of *dbf2* (PARKES and JOHNSTON 1992). Only one of these could suppress all three *dbf2 ts* alleles and was identified as *SPO12* (PARKES and JOHNSTON 1992). An 0.8-kb *Eco*RI DNA fragment containing *SPO12* (MALAVASIC and ELDER 1990) was subcloned into the multicopy vector YEp24 and introduced into both *dbf2* and *dbf2 dbf20*Δ haploid strains. Even in the presence of multiple copies of *SPO12*, which suppressed all *dbf2* strains, the *dbf2dbf20*Δ strains remained *ts* (Table 3). Thus, the outcome of both predictions was fulfilled; increasing the amount of *SPO12* so that it was no longer a limiting factor suppressed all the *dbf2* mutations by an interaction with *DBF20*.

Suppression of *dbf2* by *SPO12* is dependent on the ratio of Spo12 to Dbf2: Rather than requiring multiple copies of *SPO12*, in the *dbf2* strain J114 a single extra integrated copy of *SPO12* was sufficient to cause suppression (Figure 1). This extra copy of *SPO12* appeared to cause total suppression of *dbf2*, since the generation time of strain J114 containing either the integrated *SPO12* or the integrated *DBF2* at 37° in YPD was identical at 130 min. This result suggested that suppression of *dbf2* by *SPO12* was very sensitive to the stoichiometric balance between the Dbf2 and Spo12 proteins and that when Spo12 levels were elevated, owing to a single extra copy of the *SPO12* gene, the Dbf20 protein was able to substitute for the Dbf2 protein.

The stoichiometric relationship between Dbf2 and Spo12 can be demonstrated more rigorously in hemizygous *dbf2*/*dbf2*Δ diploids. Hemizygous *dbf2*/*dbf2*Δ diploids were constructed by crossing *dbf2* haploids with *dbf2*Δ haploids. The resulting diploids have only a *ts* version of the Dbf2 protein and might therefore be expected to be *ts*. However, they grow at 37°.

TABLE 4

Phenotype of hemizygous *dbf2*/*dbf2*Δ diploids

Relevant diploid genotype	Haploids used to make the diploids	Phenotype
dbf2 / *dbf2*Δ	J114 × S7-4B J114 × S2-2D dbf2-1 (in D273) × S7-4A bf2-2 (in D273) × S7-4A *dbf2-3* (in D273) × S7-4A	No *ts*
*dbf2 SPO12** / *dbf2*Δ *spo12*Δ	J145 × J226-1 J149 × J226-3 J149 × J226-5 J149 × J226-6	*ts*
*dbf2*Δ *spo12*Δ / *dbf2 dbf20*Δ	J113 × S7-4B J113 × J211-14C	Not *ts*
*dbf2 dbf20*Δ* / *dbf2*Δ *dbf20*Δ	J157 × J211-2C	*ts*

Diploids of the above phenotypes were made by crossing *dbf2* haploids with *dbf2*Δ haploids as listed above. In the cases of the diploid strains marked with an asterisk, one of the haploid strains used was J226-1, J226-3, J226-5, J226-6 or J221-2C. These five haploid strains contain a lethal combination of gene deletions, either *dbf2*Δ*spo12*Δ or *dbf2*Δ*dbf20*Δ, and therefore were kept alive by the plasmid pASZ11-DBF2. The diploids constructed therefore contained a plasmid-borne wild-type *DBF2* gene. Loss of the plasmid could then be observed by the production of red *ade2* sectors. These red sectors were all *ts*, whereas the white sectors, which contain the plasmid, were not *ts*.

Hemizygous diploids were constructed using all three alleles of *dbf2*, and all were found to be ts^+ (Table 4). Furthermore, hemizygous diploids become *ts* when either one copy of *SPO12* is deleted, or two copies of *DBF20* are deleted (Table 4). Thus, hemizygous *dbf2*/*dbf2*Δ diploids are ts^+ only when the ratio of *SPO12* to *DBF2* is 2:1. Consequently, in this genetic config-

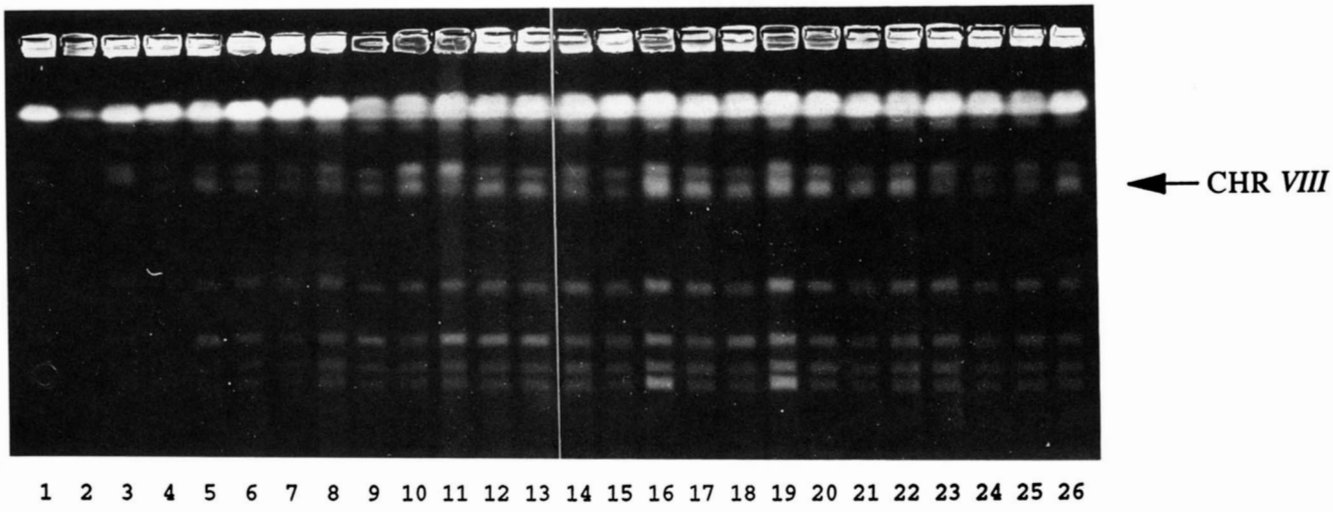

FIGURE 2.—About half of the *dbf2* revertants contain extra copies of chromosome *VIII*. The chromosomes from revertants (lanes 1 to 24, and lane 26), or from a nonreverted *dbf2* (lane 25) were visualized in a pulsed-field gel. Chromosome *VIII* is the fifth band from the bottom of the gel. It is present in an increased amount, relative to the other chromosomes, in lanes 5, 12, 13, 16, 17, 18, 19, 20, 21, 22 and 26.

uration *DBF20* functions in place of *DBF2*. Although Dbf20 is required for suppression, its stoichiometry with respect to the other proteins is not important, emphasizing that only Spo12 is a limiting factor in this system.

Half of the revertants of *dbf2* contain an extra copy of chromosome *VIII*: Although *dbf2 ts* alleles are lethal to cells at 37°, colony imprints at 37° usually develop papillae resulting from a high frequency of reversion to ts^+. Such "revertants," are shown in Figure 1. In contrast, none of the *dbf2 dbf20*Δ strains listed in Table 1 papillate at 37°. The need for a wild-type *DBF20* gene suggested that the papillae were not in fact true revertants of the mutant allele but resulted from cells in which *DBF20* was able to substitute for *DBF2*. As described above, one extra copy of *SPO12* was able to suppress *dbf2* as long as *DBF20* was present. We therefore tested the possibility that the papillating *dbf2* mutants had acquired an additional copy of *SPO12* via the acquisition of an additional chromosome *VIII*, on which *SPO12* is located. Hence, the chromosomes from 25 independent revertants of strain J114, and from a nonreverted colony of J114, were separated on a CHEF gel and stained with ethidium bromide (Figure 2). Chromosome *VIII* formed the fifth most mobile band in this gel, and 12 of these revertants had an increased amount of chromosome *VIII*.

Tandem duplication of the *SPO12* locus does not account for suppression of *dbf2*: DNA was extracted from revertants of J114, digested with *Eco*RI, and a Southern blot was made. Two radiolabeled probes were then hybridized to the blot to assess the level of chromosome *VIII* restriction fragments; and, as a control, one radiolabeled probe was hybridized to chromosome *VII* restriction fragments. For chromosome *VIII*, probes were used that hybridized to *SPO12* and *DBF3* (JOHNSTON and THOMAS 1982; J. E. SHEA, J. H. TOYN and L. H. JOHNSTON, unpublished data). For chromosome *VII*, a *DBF2* probe was used. The resulting autoradiograms (Figure 3) were quantitated by scanning densitometry and the ratios of the signal intensities were calculated (Table 5). In three of the six revertants tested, the ratio of the signal from the two chromosome *VIII* probes to the chromosome *VII* probe was approximately double the ratio found in nonreverted *dbf2* and in a wild-type strain. Since the strains used in this experiment were all haploids, the change in the ratios could not result from loss of chromosome *VII*, but only from the gain of one copy of chromosome *VIII*. Revertants that contained extra copies of the *SPO12* locus always contained extra copies of the *DBF3* locus as well, suggesting that tandem duplication of the *SPO12* locus was not a mechanism by which the high frequency of reversion occurred. This contrasts with the *CUP1* locus, which maps nearby on chromosome *VIII*, and which can increase its copy number by tandem duplication (FOGEL and WELCH 1982).

From Figure 3, it is clear that about half of the revertants contain neither an extra copy of chromosome *VIII* nor extra copies of the *SPO12* locus, and we have not determined their genetic basis. Although we have not explicitly proven that the extra copy of chromosome *VIII* causes the suppression of *dbf2*, it is almost certainly the case judging from the experiments presented above. For our purposes, the significant point is that suppression can be used as a rapid screen for chromosome acquisition, and allows us to analyze the effect of a *dbf2*-induced cell cycle arrest on chromosomal nondisjunction.

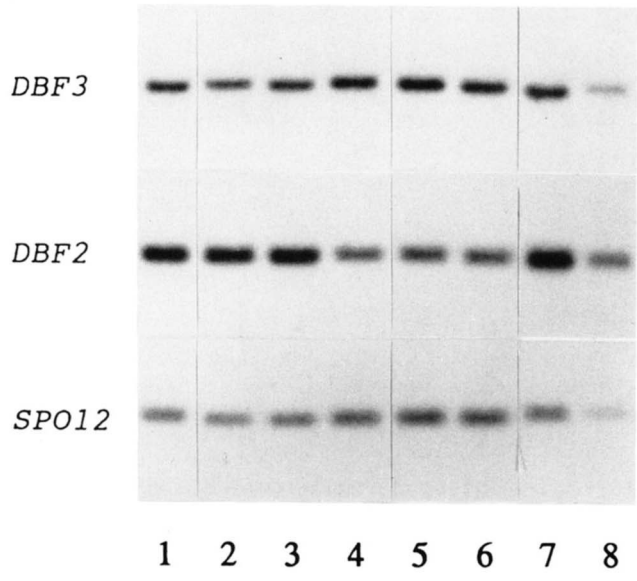

FIGURE 3.—About half of the revertants of *dbf2* contain twice the normal amount of two different genomic restriction fragments derived from chromosome *VIII*. Genomic DNA was extracted from six independent revertants and two controls, digested with *Eco*RI, and a Southern blot was made. The Southern blot was then hybridized to radiolabeled probes that recognized a 2.4-kb genomic fragment of *DBF3*, a 1.3-kb genomic fragment of *DBF2*, and an 0.8-kb genomic fragment of *SPO12*, as indicated. Hybridization was visualized by autoradiography, quantitated by scanning densitometry, and the ratios of the signal intensities were calculated (Table 5). Lanes 1–6 are revertants; lane 7 is nonreverted *dbf2*; lane 8 is the wild-type strain CG378.

The frequency of chromosome acquisition in *dbf2* mutants increases during recovery from a 37° division arrest: The frequency of chromosome *VIII* acquisition is approximately half the frequency of reversion. Thus, reversion could be used to assess the affect of *dbf2* on the frequency of chromosome acquisition. The approach used was to test for an increase in the reversion frequency after division of *dbf2* cells had been arrested by incubation at the restrictive temperature (Figure 4). Cells of strain J114 were spread on YPD plates and incubated at 37° for up to 8 hr, then returned to 25° for 1 hr, before further incubation at 37°. The colonies that grew on these plates after 4 days at 37° were then counted. As a control to test the effect of the 1-hr incubation at 25°, some plates were kept at 37° throughout the experiment, and the colonies were counted as before (Figure 4, data marked with an asterisk). The maximum frequency of reversion obtained in this experiment was 2.5×10^{-3} per cell plated, or 5×10^{-3} per survivor, sixfold and 12-fold increases over the background level of reversion, respectively. This background level of reversion resulted from cells that were already ts^+ before incubation at 37°.

Revertants that occurred as a result of the incubation at 37° became ts^+ only when given a recovery period at 25°. The need for a recovery period can be explained in terms of mis-segregation of chromosome *VIII*. For a cell to give rise to reverted progeny, it must contain an increased Spo12/Dbf2 ratio. This ratio increases only after the mis-segregated chromosomes have been partitioned into the daughter cells. Partitioning into daughter cells does not occur unless *dbf2*-arrested cells are released from their division arrest. Thus, the increase in reversion frequency required two conditions; first, the arrest of cell division at 37°, during which the events necessary for chromosomal mis-segregation took place; and, second, the recovery period at 25°, during which mis-segregated chromosomes were partitioned into the daughter cells. In terms of the frequency per cell division, the mis-segregation of chromosome *VIII* had occurred at a maximum frequency of greater than 10^{-3}. This can be compared with the wild-type frequency of chromosome *VIII* aneuploidy of about 6×10^{-6} per cell division (WHITTAKER *et al.* 1988).

TABLE 5

Ratios of the band intensities in Figure 3

	Lane							
Probes	1	2	3	4	5	6	7	8
DBF3/DBF2	0.60	0.56	0.55	*1.39*	*1.31*	*1.17*	0.54	0.44
SPO12/DBF2	0.45	0.35	0.38	*0.95*	*0.97*	*0.96*	0.38	0.34

The autoradiograms in Figure 3 were quantitated by scanning densitometry and the ratios of the signal intensities were calculated. The ratios shown in italics are approximately twice the normal values.

***dbf2* causes chromosome loss:** In principle, an increase in chromosome copy number may take place by two mechanisms; either overreplication of the DNA, presumably during S phase, resulting in extra chromosomes, or by nondisjunction of sister chromatids during M phase, resulting in aneuploid progeny. When nondisjunction occurs, every chromosome gain event is accompanied by a chromosome loss event in the sister cell, *i.e.*, one cell's gain is another cell's loss. Thus, if nondisjunction is the cause of chromosome gain in *dbf2*, we would expect to find that *dbf2* also causes an increased frequency of chromosome loss.

Chromosome loss can be assayed in diploids by constructing a strain that is heterozygous for recessive genetic markers linked on opposite arms of a chosen chromosome (HARTWELL and SMITH 1985). In this case we assayed loss of chromosome *V* by constructing diploids that are simultaneously heterozygous for *ura3* and *his1*, these two markers being situated on opposite arms of one of the two copies of chromosome *V* present in the diploid. Loss of the copy of chromosome *V* that contains the wild-type genes leaves behind the chromosome *V* containing the auxotrophic markers, resulting in a hemizygous diploid strain that is simultaneously Ura^- and His^-. In contrast, recombi-

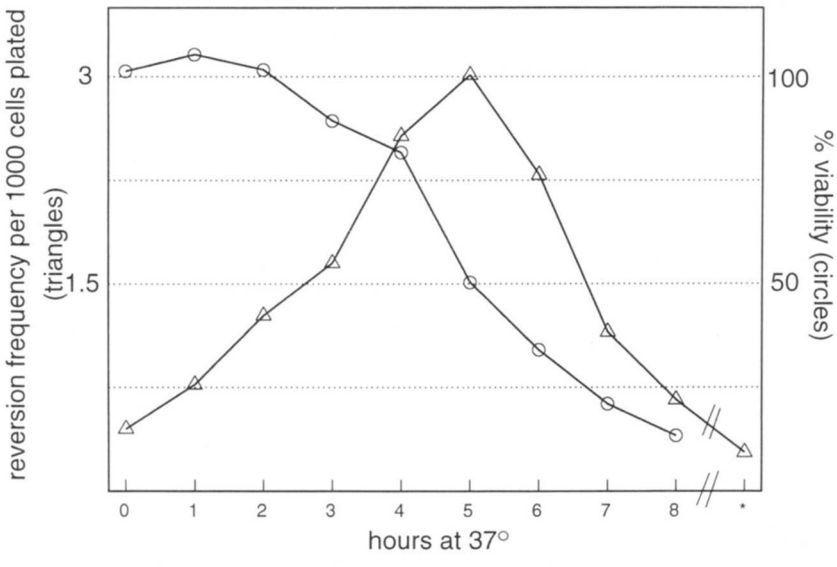

FIGURE 4.—Reversion of *dbf2* increases when division-arrested cells are allowed to recover at the permissive temperature. A single colony of strain J114, a *dbf2* haploid (see Table 1) was resuspended in saline, sonicated to obtain separate cells, and spread on YPD plates at a concentration of 10^5 cells per plate to assess the frequency of reversion, and at 200 cells per plate to estimate cell viability. The plates were then incubated for between 0 and 8 hr at 37°, followed by a 1-hr recovery period at 25°. For estimation of the reversion frequency, the plates containing 10^5 cells were incubated for a further 4 days at 37°. The number of colonies that grew were then counted. For estimation of cell viability, the plates that contained 200 cells were incubated for a further 3 days at 25° before the colonies were counted. The frequencies of reversion given in the diagram are per 1000 cells originally plated. * One plate, containing 10^5 cells, was incubated right from the beginning of the experiment for four days at 37° (*i.e.*, without any time at 25°) and the number of revertants counted.

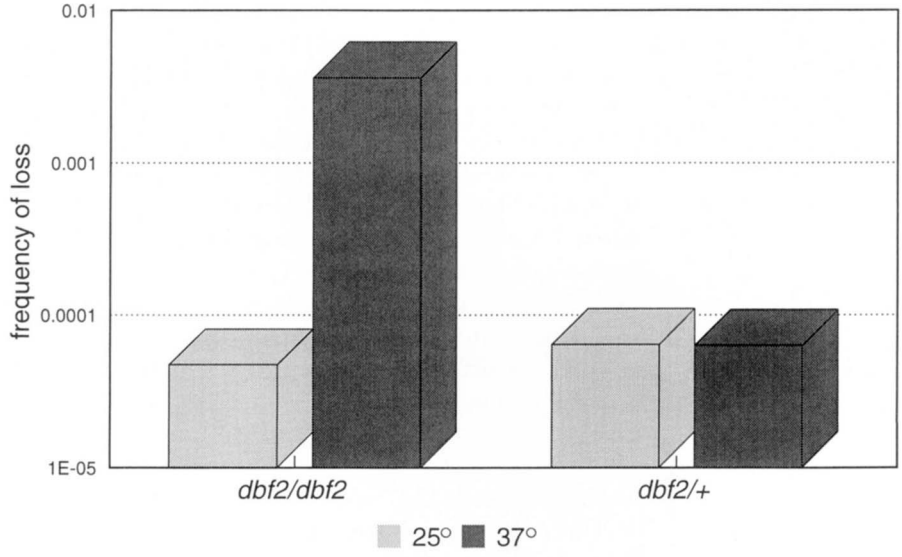

FIGURE 5.—*dbf2* causes chromosome loss. Three colonies each of J2 (*dbf2* homozygous diploid) and J1 (the isogenic heterozygote) were picked from YPD plates and grown overnight on minimal agar without supplements at 25° in order to minimize the proportion of Ura$^-$ clones at the beginning of the experiment. The chromosome *V* loss assay (see MATERIALS AND METHODS) was carried out after incubation on YPD plates for 4 hr at either 25° or 37°. The frequencies of loss were calculated per viable yeast cell. The results are shown on a $\log_{10}$ scale (1E-05 = 0.00001).

nation or mutation would result in predominantly Ura$^-$His$^+$ and Ura$^+$His$^-$ colonies. Two such diploids were tested for loss of chromosome *V*, strain J2, which was homozygous for *dbf2*, and an isogenic strain, J1, which was heterozygous for *dbf2*. The frequency of both chromosome *V* loss (URA$^-$His$^-$) and other events leading to Ura$^-$His$^+$ was assessed after incubating the cells for a period of 4 hr at either 25° or 37° (Figure 5). A 100-fold increase in the frequency of chromosome *V* loss occurred in the homozygous *dbf2* strain when it had been incubated for a 4-hr period at 37°. There was no detectable effect on the frequency of recombination (data not shown). In contrast, no increase in the frequency of chromosome loss was found in the heterozygous diploid at 37°, indicating that chromosome loss was caused by lack of function in the Dbf2 protein or the cell cycle arrest.

Similar experiments were carried out using an assay for loss of chromosome *III*, in which the markers on opposite arms were the *MAT* locus and the *LEU2* locus. Loss of chromosome *III* was quantitated by counting the frequency of *MATa leu2* clones. All three *ts* alleles of *dbf2* caused increased levels of chromosome loss (data not shown). Thus, it seems likely that the inheritance of all chromosomes would be affected by *dbf2*. In contrast to the high frequency of chromosome loss caused by *dbf2 ts* alleles, *dbf2*Δ had no significant effect on the fidelity of chromosome inheritance. The median value (of 20 tests) of a fluctuation analysis for expression of a recessive marker on chromosome *VII* was 8×10^{-6} per cell division, similar to the rate of chromosome loss and recombination in a wild-type control (HARTWELL and SMITH 1985). Thus, it was the cell cycle block, rather than the lack of *DBF2 per se*, that was the immediate cause of the chromosome loss.

DISCUSSION

The genetic interaction of *DBF2*, *DBF20* and *SPO12*: Our results suggest that Dbf2 and Dbf20 are protein kinases with overlapping activities and share the Spo12 protein as a regulatory subunit. This hypothesis stems from the observation that either, but not both *DBF2* and *DBF20* can be deleted, even though point mutations in the *DBF2* gene, which equally lead to a loss of function, are lethal. It follows that it is the presence of a nonfunctional mutant Dbf2 protein that interferes with and blocks the function of *DBF20*, rather than the lack of kinase activity *per se*. One way in which a mutant nonfunctional Dbf2 protein could block the function of the structurally very similar, but wild-type, Dbf20 protein, is to have a higher affinity for a limiting factor that is required for the vital function of Dbf20. All the data suggest that Spo12 encodes this limiting factor. There are at least six lines of evidence that are consistent with this conclusion. First, the double deletion of *DBF2* and *SPO12* is lethal (PARKES and JOHNSTON 1992), because Dbf20 requires the limiting factor (Spo12) for its vital function. In contrast, the *dbf20Δspo12Δ* double deletion is not lethal, so that *SPO12* is not required for the vital function of *DBF2*. Second, increased dosage of the *SPO12* gene suppresses *dbf2*, since under these conditions the limiting factor is no longer limiting. Third, the data suggest that recessive alleles of *dbf2* are likely to be lethal because the mutant protein sequesters the available Spo12 into a nonproductive complex; theoretically, therefore, a mutant Dbf2 protein that was unable to sequester the Spo12 would not be lethal. This is probably why increased dosage of *SPO12* is able to suppress all *dbf2* alleles (PARKES and JOHNSTON 1992). Fourth, increased dosage of *SPO12* is only able to cause suppression of *dbf2* when Dbf20 protein is available; high copy number *SPO12* does not suppress *dbf2dbf20Δ* strains. Fifth, *DBF20* is not a dosage suppressor of *dbf2* (TOYN *et al.* 1991), since it is *SPO12* that is both limiting and required for the function of *DBF20*. Furthermore, the dosage of *DBF20* has no effect in the hemizygous *dbf2/dbf2Δ* diploids. Sixth, there is some indication that the relationship between Dbf2 and Spo12 is of a stoichiometric nature. Only a single extra copy of *SPO12* is necessary to obtain full suppression of *dbf2*, not only in haploid yeast strains, but also in the unusual case of the hemizygous *dbf2/dbf2Δ* diploids. In addition, the phenotypes of the hemizygous *dbf2/dbf2Δ* diploids suggest that this is a specific effect since *SPO12* is the only gene known at present that, when present in a single extra copy, is able to suppress *dbf2*.

Further circumstantial evidence that Spo12 is a limiting factor that interacts with Dbf2 comes from the regulation of expression of their respective mRNA transcripts. First, the *SPO12* transcript is present at a very low level during mitotic cell division (PARKES and JOHNSTON 1992). In fact, it was originally thought not to be expressed in mitotic cultures (MALAVASIC and ELDER 1990). Second, both transcripts are under cell cycle regulation and accumulate to a maximum at the same stage, late in the mitotic cell cycle (PARKES and JOHNSTON 1992) and later than other cell cycle regulated transcripts. By analogy with histone gene expression, this suggests that controlled stoichiometric amounts of the proteins are required at a specific cell cycle stage.

One slightly surprising feature of these results is that the vital function of Dbf2 does not require Spo12 *i.e.*, a *spo12Δ dbf20Δ* strain is viable. We believe that this viability is due to a homolog of Spo12, or to the existence of a Spo12-like protein, that can interact with Dbf2, indeed we are searching for such a factor at present.

The involvement of *DBF2* in mitotic chromosome inheritance: It was possible to take advantage of the unusual stoichiometric relationship between *DBF2* and *SPO12* to study the role of *DBF2* in chromosome inheritance. A 2:1 ratio of *SPO12* to *dbf2* causes suppression of the *ts* phenotype of *dbf2*. Because of this, when a *dbf2* cell acquires an extra copy of chromosome *VIII*, which carries the *SPO12* locus, it will result in a suppressed clone of *dbf2* cells, and this can be readily detected by growth at 37°. Using this, we have shown that a *dbf2*-induced cell cycle block caused an increase in the frequency of chromosome acquisition, presumably as a result of nondisjunction (a 2:0 segregation of sister chromatids). Approximately half of these revertants were shown to contain an extra copy of chromosome *VIII* by pulsed field electrophoresis of intact chromosomes and Southern blot analysis of genomic restriction fragments. Thus, our conclusion that *dbf2* causes chromosome acquisition is based on direct physical evidence that revertants contain extra copies of chromosome *VIII*.

In principle the gain of chromosomes could be caused either by nondisjunction or by overreplication. However, the occurrence of overreplication in *dbf2* strains is unlikely. First, overreplication would not account for the high frequency of chromosome loss in *dbf2*, also demonstrated in this paper. Second, *dbf2* mutants carry out DNA synthesis at the normal rate, although they delay the timing of S phase, (JOHNSTON *et al.* 1990). Thus, *dbf2* affects the segregation of sister chromatids, rather than overreplicating them. Many cell cycle mutations have been shown to lower the fidelity of chromosome inheritance (PALMER, HOGAN and KOSHLAND 1990). It seems likely, therefore, that the lowering of the fidelity of chromosome inheritance in these mutants is an indirect consequence of perturbation of the cell cycle. However, a mutation could cause chromosome inheritance defects in a number of ways other than by a cell cycle mechanism. The presence of the defective protein may have an effect

per se, or it may be only the loss of function of the mutant protein that causes the effect. For *dbf2*, the presence of a nonfunctional protein has no effect *per se*, because heterozygous *dbf2/DBF2* diploids did not have a chromosome loss phenotype.

To find out whether loss of function by itself caused chromosome loss, it was necessary to measure chromosome loss when the *DBF2* gene was absent, but under conditions that did not block the cell cycle. This could be carried out for *DBF2*, because the cell cycle is not blocked in *dbf2*Δ strains. We found no evidence for an effect on chromosome inheritance in *dbf2*Δ/*dbf2*Δ diploids, confirming that it was not the lack of function in *dbf2* mutants *per se*. Thus, by themselves, neither lack of function nor the presence of a *dbf2* mutant allele could account for the chromosome loss. The remaining possibility is that the cell cycle arrest was the immediate cause, and that the relationship of cause to effect follows the route *dbf2* → cell cycle arrest → chromosome loss. It therefore seems reasonable to conclude that nondisjunction of sister chromatids occurs during the *dbf2*-induced cell cycle arrest. This may seem surprising, because the bulk of chromatin has already divided in *dbf2*-blocked cells, and therefore past the stage in the cell cycle during which nondisjunction would normally be able to take place. However, the fact that strands of chromatin connecting the mother and daughter cells can be detected in *dbf2*-blocked cells (JOHNSTON *et al.* 1990) may mean that nondisjunction is still possible during the block. This has implications for the molecular nature of the function regulated by Dbf2. Since Dbf2 is a protein kinase, it presumably regulates the function of some other protein(s) that actually carries out a physical task(s). This task would be necessary to assist efficient separation of sister chromatids after the time when separation had been initiated, namely during anaphase, after the metaphase to anaphase transition had taken place.

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