Conditional Mutations in the Yeast DNA Primase Genes Affect Different Aspects of DNA Metabolism and Interactions in the DNA Polymerase α-Primase Complex

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ABSTRACT

Different *pri1* and *pri2* conditional mutants of *Saccharomyces cerevisiae* altered, respectively, in the small (p48) and large (p58) subunits of DNA primase, show an enhanced rate of both mitotic intrachromosomal recombination and spontaneous mutation, to an extent which is correlated with the severity of their defects in cell growth and DNA synthesis. These effects might be attributable to the formation of nicked and gapped DNA molecules that are substrates for recombination and error-prone repair, due to defective DNA replication in the primase mutants. Furthermore, *pri1* and *pri2* mutations inhibit sporulation and affect spore viability, with the unsporulated mutant cells arresting with a single nucleus, suggesting that DNA primase plays a critical role during meiosis. The observation that all possible pairwise combinations of two *pri1* and two *pri2* alleles are lethal provides further evidence for direct interaction of the primase subunits *in vivo*. Immunopurification and immunoprecipitation studies on wild-type and mutant strains suggest that the small subunit has a major role in determining primase activity, whereas the large subunit directly interacts with DNA polymerase α, and either mediates or stabilizes association of the p48 polypeptide in the DNA polymerase α-primase complex.

IN the yeast *Saccharomyces cerevisiae*, as in all eukaryotic systems analyzed so far, a DNA primase activity is associated with DNA polymerase α (polα) in a four-subunit complex that appears to play a major role in initiation of DNA replication at an origin and in priming and elongation of Okazaki fragments on the lagging strand (LEHMAN and KAGUNI 1989; TSURIMOTO, MELENDY and STILLMAN 1990). The yeast complex can be separated into two fractions, one of which, containing the p48 and p58 polypeptides, is able to synthesize short RNA primers (9–11 nucleotides in length) that can be elongated by polα (PLEVANI *et al.* 1985; BROOKS and DUMAS 1989). Cloning and characterization of the *PRI1* and *PRI2* genes encoding, respectively, the p48 and p58 protein species, have shown that they are both unique in the haploid yeast genome and essential for cell viability, and that the amino acid sequence of their encoded products is highly homologous to that of the corresponding mouse primase polypeptides (PLEVANI, FRANCESCONI and LUCCHINI 1987; FOIANI *et al.* 1989b; PRUSSAK, ALMAZAN and TSENG 1989; FRANCESCONI *et al.* 1991). Earlier studies with anti-p48 and anti-p58 antibodies and an affinity labeling procedure suggest that both proteins are necessary for optimal DNA primase activity (FOIANI *et al.* 1989a). However, the inability to purify each primase subunit in an isolated form in any eukaryotic system has until now limited the possibility to use *in vitro* reconstitution studies to further analyze their specific role in determining enzyme activity and their interactions.

To address these questions and to gain some information about the function of DNA primase in different aspects of DNA metabolism, we have produced conditional *pri1* and *pri2* mutants by random mutagenesis of the cloned genes and replacement of the wild-type copy at the corresponding chromosomal locus (FRANCESCONI *et al.* 1991). Preliminary characterization of different recessive alleles, *i.e.*, the leaky and temperature-sensitive (ts) *pri1-1*, the cold-sensitive (cs) *pri1-2*, the ts *pri2-1* and the ts *pri2-2* alleles, which all carry single base-pair substitutions causing a change in amino acid residues conserved in the corresponding mouse polypeptides, has allowed us to assess the essential role of DNA primase in DNA replication *in vivo*. In fact, the primary defect in all these mutants, after shifting to nonpermissive temperature, is an impairment in DNA synthesis, ranging between a complete arrest in the *pri2-1* mutant to only a doubling of the time needed to complete one round of DNA replication in the *pri2-2* mutant. These defects are correlated with cell growth arrest or reduced growth rate, accumulation of dumbbell-shaped cells and inability to synthesize appropriate amounts of high molecular weight DNA molecules (FRANCESCONI *et al.* 1991).

TABLE 1

S. cerevisiae strains

Strain	Genotype
DGL2-1C[a]	*MATα met4 lys1*
TD28[a]	*MATa ura3-52 ino1 can1*
TS72[a]	*MATa ura3-52 ino1 can1 pri1-1*
CS33[a]	*MATa ura3-52 ino1 can1 pri1-2*
A16[a]	*MATa ura3-52 ino1 can1 pri2-1*
F402[a]	*MATa ura3-52 ino1 can1 pri2-2*
TS1-4[b]	*MATa ura3-52 ino1 can1 pol1-1*
DGL273-3D[b]	*MATa ura3-52 lys1 can1 cdc17-1*
DGL277-5B[b]	*MATα ura3-52 lys1 can1 ino1 cdc 17-2*
DGL278-9A[b]	*MATα ura3-52 lys1 ino1 hpr3*
344-109D[c]	*MATa ura3-52 trp1 ade1-101 leu2-112::URA3::leu2-k his3-513::TRP1::his3-537*
344-115B[c]	*MATα ura3-52 trp1 leu2-112::URA3::leu2-k his3-513::TRP1::his3-537*

[a] Described in FRANCESCONI *et al.* (1991).
[b] Described in LUCCHINI *et al.* (1990).
[c] Described in AGUILERA and KLEIN (1988).

We have now further characterized these mutants by studying their behavior with respect to other processes involving DNA metabolism, including mitotic recombination, spontaneous mutation frequency and meiosis, all of which might be affected by defects in the DNA replication machinery. Moreover, we report a preliminary characterization of the polα-primase complex in the different mutants that provides some new insights about protein-protein interactions among the polα-primase polypeptides and the role of the primase subunits in supporting enzyme activity.

MATERIALS AND METHODS

Strains and media: The original *pri1* and *pri2* mutant strains TS72, CS33, A16 and F402 and their isogenic wild-type strain TD28 (Table 1) were used for immunoprecipitation and immunopurification of the polα-primase complex. Strains used to determine intrachromosomal recombination rate and *CAN1* to *can1* mutation rate were constructed as follows. First, the isogenic mutant strains TS72, CS33, A16 and F402 were crossed to strain 344-115B (Table 1), to generate diploid strain DML82, DML83, DML84, DML85, respectively. One *pri1-1*, *pri1-2*, *pri2-1* or *pri2-2* meiotic segregant from each of these diploids was then crossed to strain 344-109D (Table 1) to obtain, respectively, the diploid strains DML150, DML148, DML96 and DML102. Strains with the appropriate genotype were chosen among the meiotic segregants from this second round of diploids to carry on the two assays described in the next section. *CAN1 pol1-1* and *CAN1 cdc17-1* strains were obtained as meiotic segregants from crosses TS1-4 to 344-115B and DGL273-3D to 344-115B, respectively, while *CAN1 hpr3* and *CAN1 cdc17-2* segregants were derived from crosses DGL278-9A to 344-109D and DGL277-5B to 344-109D, respectively (Table 1). Strain TS1-4 is isogenic to TD28, while strains DGL273-3D, DGL278-9A and DGL277-5B are strictly related to TD28 (LUCCHINI *et al.* 1990). Diploid strains for sporulation analysis were constructed by crossing first TS72, A16 and F402 to DGL2-1C (Table 1). *MATα pri1-1*, *MATα pri2-1* and *MATα pri2-2* meiotic segregants from the obtained diploid strains were then back-crossed to TS72, A16 and F402, respectively, to originate the corresponding *pri1/pri1* or *pri2/pri2* strains, and to TD28 to give rise to *PRI1/pri1* or *PRI2/pri2* strains. Rich medium YPD, synthetic medium SD and synthetic complete medium with amino acids and bases omitted as specified, were as described by SHERMAN, FINK and HICKS (1986). L-Canavanine sulfate was added to synthetic complete medium lacking arginine at the final concentration of 30 μg/ml. Sporulation media and conditions were as described by LUCCHINI *et al.* (1978).

Fluctuation test: For the recombination assay we used four *pri1-1*, four *pri2-1*, three *pri2-2*, one *pri1-2* and four *PRI1 PRI2* (wild-type) strains constructed as described above, which were all Leu$^-$ His$^-$ Ura$^+$ Trp$^+$ and were shown by Southern analysis to carry both the *LEU2* and the *HIS3* duplications as depicted in Figure 1A. Analysis of the *CAN1* to *can1* mutation rate was performed on three *CAN1 pri1-1*, one *CAN1 pri1-2*, three *CAN1 pri2-1*, one *CAN1 pri2-2*, one *CAN1 pol1-1*, one *CAN1 cdc17-1*, one *CAN1 cdc17-2*, one *CAN1 hpr3* and 10 *CAN1 PRI1 PRI2 POL1* (wild-type) strains. According to the procedure described by AGUILERA and KLEIN (1988), with minor modifications, single cells were allowed to form colonies (approximately 2–3 × 10^7 cells/colony) on YPD plates at 25° (wild-type, *pri1-1*, *pri1-2* and *pri2-1* strains), 28° (wild-type and *pri2-2* strains for the recombination test) or 37° (wild-type and *pri2-2* strains for the mutation test). Growth temperatures were chosen as the ones providing the higher recombination or mutation rates in the mutants, based on preliminary tests. Five to eight independent colonies for each strain were resuspended in 1 ml of water and plated on selective SD −His and SD −Leu media (approximately 3 × 10^5 cells/plate) for the recombination test, on SD + canavanine (approximately 1 × 10^7 cells/plate) for the mutation test, and on complete synthetic medium (approximately 3 × 10^2 cells/plate) to determine the exact viable title of each cell suspension. Plates were incubated at 25° and colonies were counted after 5 days. Leu$^+$ or His$^+$ recombinant clones were then replicated on SD −Ura and SD −Trp, respectively, to distinguish between reciprocal recombination and gene conversion events. Recombination and mutation rates (recombinants or mutants/cell/generation) were calculated for each strain according to LEA and COULSON (1948). No significant differences were observed in either recombination or mutation rates among different wild-type strains, even if pregrown at different temperatures, or among different strains carrying the same *pri1* or *pri2* conditional allele.

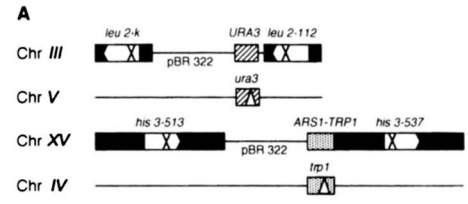
Biochemical methods: Preparation of yeast crude extracts, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE), Western blot analysis, and immunochromatographic purification of the polα-primase complex with the y48 monoclonal antibody (Mab) covalently coupled to Protein A-Sepharose 6B (Pharmacia) were as previously described (PLEVANI *et al.* 1985). Immunoprecipitation of the polα-primase complex from wild-type and mutant cells was achieved by adsorption for 2 hr at 4° of 25 mg of protein in the corresponding extracts to 0.15 ml of a 1:1 dilution of Mab y48-Protein A-Sepharose 6B in breaking buffer (50 mM Tris-HCl at pH 8.0, 100 mM NaCl, 0.5 mM EDTA, 5 mM $MgCl_2$, 1% dimethyl sulfoxide, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml of pepstatin A, leupeptin and chymostatin). After centrifugation for 2 min in an Eppendorf centrifuge, the resin has been washed three times with 5 ml of breaking buffer and divided in several aliquots, corresponding to 2 mg of protein in the initial extracts. Each aliquot was then resuspended in 0.080 ml of

DNA polymerase or DNA primase reaction mixtures and enzymatic activities in the immunoprecipitates were measured as previously described, with [^{3}H]dATP (750 cpm/pmol) as the labeled substrate (PLEVANI *et al.* 1985). The immunological reagents used for Western blot analysis to detect polα were a mixture of anti-polα Mabs (LUCCHINI *et al.* 1985). Affinity purified rabbit polyclonal antibodies raised against trpE-PRI2 or trpE-PRI1 fusion proteins, produced in *Escherichia coli* and containing, respectively, the carboxyl-terminal 419 amino acids of p58 or the carboxyl-terminal 307 amino acids of p48 were used to detect p58 or p48 on Western blots. These antibodies specifically recognize either p58 or p48 on Western blots of both immunopurified polα-primase complex and yeast crude extracts (SANTOCANALE *et al.* 1993; this study).

RESULTS

Conditional *pri1* and *pri2* mutations lead to enhanced rates of intrachromosomal mitotic recombination and mutation: An increase in the rate of mitotic recombination and mutation has been observed as a consequence of ts mutations in a number of DNA synthesis genes, including *CDC9* (DNA ligase) (GAME, JOHNSTON and VON BORSTEL 1979), *CDC8* (thymidylate kinase), *POL1/CDC17* (DNA polymerase α), and *POL3/CDC2* (DNA polymerase δ) (HARTWELL and SMITH 1985; AGUILERA and KLEIN 1988).

To determine whether defects in the DNA primase subunits would also affect mitotic recombination, we used a genetic system capable of measuring both gene conversion and reciprocal recombination events, previously described by AGUILERA and KLEIN (1988). Briefly, the system is based on haploid strains carrying heteroallelic duplications of the *LEU2* gene on chromosome III and of the *HIS3* gene on chromosome *XV* (Figure 1A). The *URA3* marker is located between the *leu2-112* and the *leu2-k* alleles, while the *TRP1* marker separates the *his3-513* and the *his3-537* alleles, and the corresponding chromosomal loci carry mutant *ura3* and *trp1* alleles, respectively. Recombinants are selected as Leu$^+$ or His$^+$ clones, which are then analyzed by replica plating to distinguish between different recombination events involving the repeated sequences at each locus: (i) reciprocal recombination, that leads to loss of the *URA3* or *TRP1* marker (Leu$^+$ Ura$^-$ and His$^+$ Trp$^-$ clones) and (ii) gene conversion events (Leu$^+$ Ura$^+$ and His$^+$ Trp$^+$ clones). The duplications were introduced into a *pri1* or *pri2* background by two rounds of crosses (see MATERIALS AND METHODS) involving the isogenic mutant strains and two wild-type strains carrying the duplications, which are genetically related to each other and show nearly identical rates of mitotic intrachromosomal recombination and spontaneous *CAN1* to *can1* mutation (AGUILERA and KLEIN 1988; our unpublished observation). The second round of crosses gave rise to diploid strains DML150, DML148, DML96 and DML102, that were heterozygous for one *pri1* or *pri2* allele and homozygous for the duplications. At least



B

	Samples[a]	Rate (x10^{-6})[b] His$^+$	Rate (x10^{-6})[b] Leu$^+$	Leu$^+$ Ura$^+$/Ura$^-$	His$^+$ Trp$^+$/Trp$^-$
wild-type	26 [25°-28°]	7.5	12.4	1.1	1.3
pri 1-1	22 [25°]	161 (x21)	445 (x36)	1.9	2.5
pri 1-2	8 [25°]	181 (x24)	306 (x25)	1.5	3.5
pri 2-1	29 [25°]	137 (x18)	73 (x6)	1.1	1.6
pri 2-2	20 [28°]	34 (x4.5)	25 (x2)	1.1	1.6

FIGURE 1.—Experimental scheme to measure the effect of *pri1* and *pri2* mutations on intrachromosomal recombination. (A) The common basic genetic features of all the strains used for this study are shown (see text for details). (B) The rate of recombination at *LEU2* and *HIS3* in wild-type, *pri1* and *pri2* strains is given, as well as the ratio (Ura$^+$/Ura$^-$ and Trp$^+$/Trp$^-$) between gene conversion and pop-out events at each locus.

[a] Total number of independent clones analyzed for each *pri1* or *pri2* allele. Growth temperature is indicated within brackets.

[b] Each entry represents the average value of the rates obtained for each different strain carrying the same *pri1* or *pri2* alleles according to the fluctuation test of LEA and COULSON (1948). The standard error was always <20%. Numbers in parenthesis indicate increase over the wild-type rate of recombination.

10 tetrads from each of them were analyzed and we observed a 2:2 segregation of high frequency of Leu$^+$ and His$^+$ papillae in all the tested tetrads. This hyper-recombination phenotype always cosegregated with the *pri1* or *pri2* mutations, as exemplified in Figure 2 for the *pri1-1* and *pri2-1* alleles, and it was not observed in tetrads from *PRI1/PRI1 PRI2/PRI2* control strains. These data indicated that the observed hyper-recombination phenotype was due to the primase mutations under analysis. To quantify the effect of the *pri1* and *pri2* alleles on recombination, we performed a fluctuation test as described in MATERIALS AND METHODS. As shown in Figure 1B, all of the mutations, to different extents, cause a significant enhancement in recombination rates at both loci. Quantitative differences among different mutants are positively correlated with the effect of the mutations on growth rate at the temperature used for the recombination assay (FRANCESCONI *et al.* 1991). Thus, the ts *pri1-1* strains and the cs *pri1-2* strains show the highest recombination rate (20–30-fold increase over the wild-type rate) and grow more slowly at 25° than the ts *pri2-1* strains (6–18-fold increase). In addition, the growth rate at 28° is nearly wild-type in the ts *pri2-2* strains, which show only a modest 2–4-fold enhancement in recombination rate. We observed an increase in both reciprocal recombination and gene conversion events in all the mutants (Figure 1B). Whereas the ratio of the frequencies for the two kinds of recom-

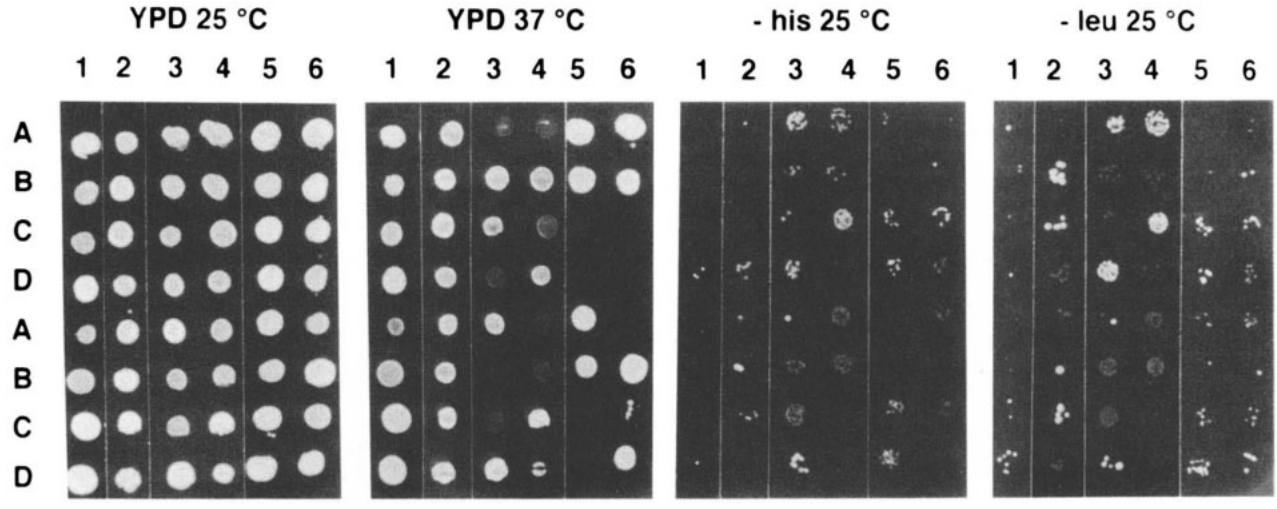


FIGURE 2.—The *pri1-1* and *pri2-1* ts phenotype is associated with high frequency of recombination. Tetrads from the diploid strains DML126 *PRI1/PRI1 PRI2/PRI2* (lanes 1 and 2), DML150 *pri1-1/PRI1 PRI2/PRI2* (lanes 3 and 4) and DML96 *PRI1/PRI1 pri2-1/PRI2* (lanes 5 and 6), all carrying homozygous *LEU2* and *HIS3* duplications, were dissected after sporulation and analyzed by spotting about 10^5 cells for each segregant clone on plates containing the indicated media. Strain DML126 was obtained by crossing one *PRI1* meiotic segregant from DML82 to strain 344-109D. Pictures were taken after 36 hours of incubation at 25° or 37° for YPD plates and after three days of incubation at 25° for SD −Leu and SD −His plates. Growth on SD complete medium was comparable to growth on YPD at the same temperatures. Two tetrads are shown in each lane and letters A, B, C, D indicate four spores from the same tetrad.

bination events are very similar in wild-type and *pri2* strains, both *pri1* mutations seem to favour gene conversion, since the difference compared to wild type was significant at $P < 0.01$ by χ^2 analysis.

Mutations affecting mitotic recombination often have been observed to cause a mutator phenotype (HARTWELL and SMITH 1985; AGUILERA and KLEIN 1988). For this reason, we analyzed the effect of *pri1* and *pri2* mutations on spontaneous rate of mutation of the wild-type *CAN1* gene to *can1* alleles that confer resistance to the arginine analogue canavanine, and a fluctuation test was performed as described in MATERIALS AND METHODS. As a control, four different ts *pol1* alleles (*pol1-1*, *hpr3*, *cdc17-1* and *cdc17-2*) were assayed in parallel. As summarized in Table 2, all the mutants, but one, showed a 6–10-fold enhancement of *CAN1* to *can1* mutation rate compared to wild type, when colonies were grown at 25° before plating on selective medium. Only the weak ts *pri2-2* strain had to be grown at 37° to observe any increase in the mutation rate. Because the *CAN1* to *can1* mutation rates were identical in wild-type strains grown at 25° or at 37°, the 4-fold increase found in the *pri2-2* strain can be attributed to the mutation in the *PRI2* gene.

***pri1* and *pri2* ts mutations interfere with meiosis and spore survival:** The essential role of polα in premeiotic DNA synthesis has been clearly demonstrated (BUDD *et al.* 1989). It was therefore of interest to determine whether the associated DNA primase, essential for mitotic DNA synthesis, was also involved in the meiotic process. For this purpose we compared sporulation frequencies and spore survival in isogenic diploid strains (see MATERIALS AND METHODS), carrying each ts *pri1* and *pri2* recessive mutation in the heterozygous or homozygous state. Cells were grown in YPD at 25° and then transferred to liquid sporulation medium at 25° or 28° (LUCCHINI *et al.* 1978). Samples for DAPI staining of the nuclei were taken at different times and sporulation frequency and spore survival were determined after three days.

As summarized in Table 3, sporulation is severely affected in *pri1-1* and *pri2-1* homozygous strains even at 25°, a temperature that either does not alter (*pri2-1*) or causes only a slight delay (*pri1-1*) in the rates of mitotic growth and DNA synthesis (FRANCESCONI *et al.* 1991). The *pri2-2* homozygous strain also exhibits a less severe defect in sporulation, as expected from

TABLE 2

Mutator phenotype of DNA primase mutants

Strain	Temperature (°C)	Samples[a]	*CAN1* to *can1* mutation rate ($\times 10^{-7}$)[b]
Wild-type	25–37	80	1.0
pri1-1	**25**	**24**	**7.5**
pri1-2	**25**	**16**	**10.0**
pri2-1	25	32	6.7
pri2-2	37	8	4.3
pol1-1	25	8	7.7
hpr3	**25**	**8**	**6.1**
cdc17-1	**25**	**8**	**7.0**
cdc17-2	25	8	3.3

[a] Total number of independent clones analyzed for each *pri1* or *pri2* allele.

[b] Each entry represents the average value of the rates obtained for each different strain carrying the same *pri1* or *pri2* allele, measured by the fluctuation test of Lea and Coulson (see MATERIALS AND METHODS). Standard error was always <20%.

TABLE 3

Effect of *pri1* and *pri2* mutations on sporulation and spore survival

Strain	Sporulation frequency (%) 25°	28°	No. of dissected tetrads[a]	No. of tetrads with viable spores[b] 4	3	2	1
PRI1/pri1-1	35	30	8	7		1	
pri1-1/pri1-1	8	1.5	7	2	3	2	
PRI2/pri2-1	36	31	8	7		1	
pri2-1/pri2-1	5	0	9	2	2	1	4
PRI2/pri2-2	28	29	8	7	1		
pri2-2/pri2-2	20	14	8	3	3	2	

[a] Tetrads were dissected from the 25° sporulated cultures.
[b] Spore viability was assayed at 25° on YPD.

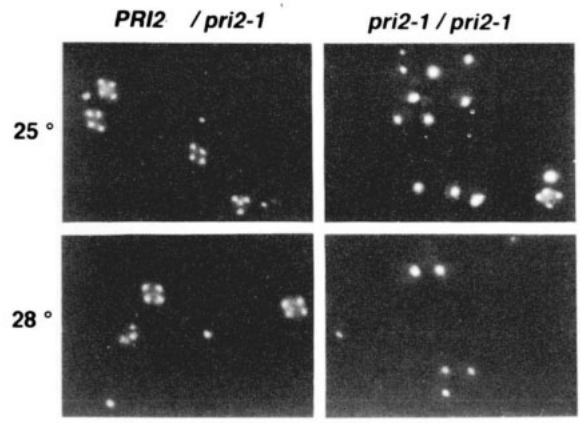


FIGURE 3.—DAPI stained nuclei in sporulating wild-type and *pri2-1* diploid cells. Samples for staining with 4′,6-diamidino-2-phenylindole (DAPI; TRUEHEART, BOEKE and FINK, 1987) were collected 48 hours after cell transfer to sporulation medium (see text for details).

the lesser effect of this mutation on cell growth and DNA synthesis. All the unsporulated cells in the homozygous mutants arrested with a single nucleus, as illustrated for the *pri2-1/pri2-1* strain in Figure 3. Moreover, the appearance of asci was delayed by more than 12 hr for the homozygous strains compared to the corresponding heterozygous strains at 25° (data not shown), and a significant number contained inviable spores (Table 3). The latter finding suggests that, even when meiosis and sporulation take place, some meiotic products are genetically defective.

Mutations in the *PRI1* and *PRI2* genes differently affect protein-protein interactions: Since p48 and p58 polypeptides interact with each other, it could be expected that specific missense mutations in one of the two genes would suppress certain missense mutations in the other gene. Alternatively, combinations of conditional mutations in both genes might result in a more dramatic defect than would the single mutations. In order to address this question, we crossed the *pri1* and *pri2* conditional mutants in all the possible pairwise combinations and analyzed the phenotype of double *pri1* and *pri2* segregants from these crosses. As summarized in Table 4, with the only exception of rare *pri1-2 pri2-2* segregants, which were severely affected in growth rate at any temperature, all the other double *pri1 pri2* combinations had a lethal effect. These data add further evidence for *in vivo* interaction between the two subunits.

A parallel biochemical approach allowed us to analyze the effect of these mutations on the stability of the corresponding gene products and on their ability to interact with one another and with polα. Immunoblot analysis of mutant crude extracts (Figure 4) shows that *pri1-1* and *pri2-1* alleles encode very labile polypeptides that are barely detectable using our antibodies, whereas the *pri1-2* mutation does not significantly affect the level of the corresponding polypeptide and p58 level is significantly lower than in wild-type in *pri2-2* cells. These differences in protein stability correlate well with the effect of the mutations on growth rate and DNA synthesis. The level of the other wild-type primase subunit was substantially unaffected in all the mutant extracts.

To analyze the effect of the mutations on the interactions among the polα-primase complex polypeptides, we applied an immunochromatographic procedure, based on the use of the anti-polα Mab y48, which allows the purification of the polα-primase complex from wild-type cells (PLEVANI *et al.* 1985). With this procedure, we immunopurified polα and the associated polypeptides from the above *pri1* and *pri2* mutant extracts. As shown in Figure 5, the amount of p48 associated with polα is severely reduced in *pri1-1* preparations, in accord with the increased lability of the mutated polypeptide (Figure 4), while the ratio between the amount of p58 and polα polypeptides is similar to the wild-type preparation. In contrast, the amounts of both the mutated p58 and the wild-type p48 associated with polα in *pri2-1* are both highly reduced, even though the level of p48 was normal in the crude extract (Figure 4). Similarly, the amount of polα-associated wild-type p48 in *pri2-2* preparations is reduced from the wild-type and seems to be determined by the amount of mutant p58 recovered.

To further analyze the role of the two subunits in enzyme function, we tried to correlate primase activity with the variable levels of the primase polypeptides observed in wild-type, *pri1-1* and *pri2-1* complexes. Towards this end, we used the anti-polα y48 antibodies, which do not inhibit either DNA polymerase or primase activity (PLEVANI *et al.* 1985), to immunoprecipitate polα from wild-type and mutant extracts. The immunoprecipitates were analyzed directly for DNA polymerase and primase activities and by Western blotting.

The polypeptide composition of the immunoprecipitates was similar to that observed after immunochromatography, although, under these conditions,

TABLE 4

Lethality of *pri1 pri2* double mutants

Strain	No. of dissected tetrads[a]	No. of tetrads with viable spores[b]		
		4	3	2
$\frac{PRI1\ pri2\text{-}1}{pri1\text{-}1\ PRI2}$	10	2 (PD)	6 (TT)	2 (NPD)
$\frac{PRI1\ pri2\text{-}2}{pri1\text{-}1\ PRI2}$	9	1 (PD)	5 (TT)	3 (NPD)
$\frac{PRI1\ pri2\text{-}1}{pri1\text{-}2\ PRI2}$	8	2 (PD)	4 (TT)	2 (NPD)
$\frac{PRI1\ pri2\text{-}2}{pri1\text{-}2\ PRI2}$	10	3 (1PD + 2TT)	4 (3TT + 1NPD)	3 (NPD)

Tetrads were defined as parental ditypes (PD), nonparental ditypes (NDP) or tetratypes (TT) based on segregation of the ts or cs phenotypes, that were shown to correlate with the presence of *pri1* or/and *pri2* mutations by a complementation test. From this analysis, all the expected *pri1 pri2* double mutants were unviable, with the only exception of three *pri1-2 pri2-2* segregants, which gave rise to very small colonies.

[a] Tetrads were dissected from the 25° sporulated cultures.

[b] Spore viability was assayed at 25° on YPD.

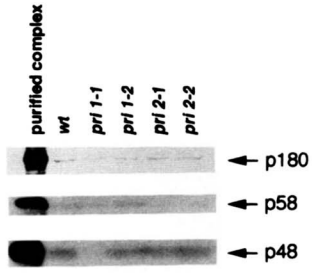


FIGURE 4.—Western blot analysis on crude extract from wild-type and *pri1* or *pri2* cells. Crude extracts were prepared from cell cultures grown in YPD at 25°. Each lane represents 100 μg of the indicated crude extract, analyzed by SDS-PAGE, transferred to nitrocellulose filters and probed, respectively, with a mixture of anti-polα Mabs (top row), or with anti-p58 (middle row) or anti-p48 (bottom row) polyclonal antibodies (see MATERIALS AND METHODS). The identity of the bands in the crude extracts is assessed by comigration with the corresponding polypeptides in the immunopurified polα-primase complex, that was run on the same gel.

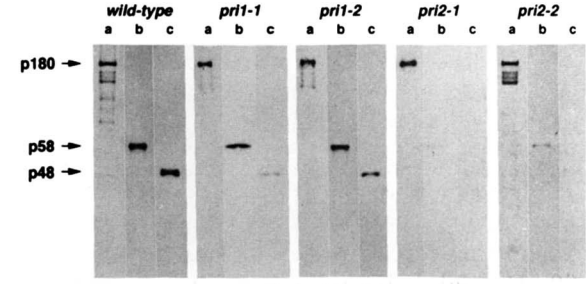


FIGURE 5.—Western blot analysis of proteins purified from wild-type, *pri1* or *pri2* cell extracts by anti-polα monoclonal antibodies. Immunochromatography with Mab-y48 was performed on the crude extracts described in Figure 4. Polypeptides present in 0.015 ml samples of each Mab eluate were separated by SDS-PAGE, transferred to nitrocellulose filters and probed, respectively, with a mixture of anti-polα monoclonal antibodies (lanes a), or with anti-p58 (lanes b) or anti-p48 (lanes c) polyclonal antibodies.

the recovery of the p48 polypeptide from both mutant extracts was somewhat higher relative to the level of p58 than that shown in Figure 5, but still highly reduced compared to wild-type levels. As summarized in Table 5, the amount of DNA polymerase activity was comparable in wild-type and mutant immunoprecipitates, in agreement with the wild-type level of polα polypeptide observed on Western blots for each preparation. Due to the very low amount of DNA primase in these immunoprecipitates, the efficiency of the direct assay measuring the synthesis of labeled oligo(rA) primers on a poly(dT) template (PLEVANI *et al.* 1985) was below the level of detection. Therefore we employed the more sensitive combined assay measuring *E. coli* DNA polymerase I activity as a function of primer formation by yeast DNA primase. Under these assay conditions (Table 5), DNA primase activity was reduced to 50–60% of the wild-type level in the mutants when incubation was performed at the temperature permissive for the mutants (25°). *E. coli* DNA polymerase I works poorly at 25°. When the same assay was performed at 37° to optimize elongation of RNA primers by *E. coli* polymerase, primase activity in the mutants was found to be reduced to 6–11% of the wild-type level. To confirm that this 5-fold reduction of primase activity at nonpermissive temperature was due to a lower efficiency of primer formation, a two-step reaction was carried out, where primer formation was allowed to occur for 20 min. at 25° or 37°, before elongation by *E. coli* DNA polymerase I could take place. Under these conditions, primase activity in the mutant immunoprecipitates was reduced by preincubation at nonpermissive versus permissive temperature, that we attribute to temperature-sensitivity of the primase enzyme. The two-step reaction comparison also indicates that primase activities measured in either mutant cannot be considered as background levels. In fact, residual activity is de-

TABLE 5

DNA polymerase and DNA primase activity in immunoprecipitates

Immunoprecipitate	DNA polymerase activity (cpm) at 25°	DNA primase activity (cpm)			
		One-step reaction[a]		Two-step reaction[b]	
		25°	37°	25° → 37°	37° → 37°
Wild-type	37,118	2,203	173,990	84,499	179,950
pri1-1	37,146	1,157	20,160	8,107	3,440
pri2-1	30,276	1,335	10,035	10,123	3,170

Numbers given in the table represent the average value of two independent assays on each immunoprecipitate. Standard error was always <10%.

[a] DNA primase activity was assayed by incubating directly the reaction mixture, including *E. coli* DNA polymerase I, at the indicated temperatures for 1 hr.

[b] *E. coli* DNA polymerase I and [^{3}H]dATP were added after preincubation of the reaction mixture for 20 minutes at 25° or 37° and the reaction was allowed to proceed at 37° for 1 hr.

tectable for the 25° to 37° condition, that is instead reduced by incubation at 37°, whereas activity actually increases for the wild-type immunoprecipitate when pre-incubated at 37° *vs.* 25°.

DISCUSSION

Initiation of DNA replication at an origin and discontinuous DNA synthesis on the lagging strand require a primase-mediated RNA priming mechanism (KORNBERG and BAKER 1991). In eukaryotes, DNA primase activity is associated with a structurally and functionally highly conserved heterodimer of 50 kDa and 60 kDa, tightly bound to polα. Its essential function in mitotic DNA replication has been clearly assessed by *in vitro* experimental evidence (LEHMAN and KAGUNI 1989; KORNBERG and BAKER 1991), and *in vivo* characterization of yeast *pri1* and *pri2* mutants carrying conditional point mutations in the genes coding, respectively, for the small (p48) and large (p58) primase subunits (FRANCESCONI *et al.* 1991). To determine whether the primase polypeptides have specific roles in other aspects of DNA metabolism, we have tested the ability of two *pri1* and two *pri2* conditional mutants to influence rates of mitotic intrachromosomal recombination, spontaneous mutation and the efficiency of meiosis. In all the *pri1* and *pri2* strains we tested, the recombination rate was significantly higher than in wild-type strains, similar to what was observed previously for different *pol1* mutants (LUCCHINI *et al.* 1990). Although more *pri1* and *pri2* alleles should be analyzed, the differences in the extent to which the recombination rate is increased among the mutants seem to be more allele-specific than gene-specific, and to be correlated with the severity of the defects in growth rate and DNA synthesis. Both reciprocal recombination and gene conversion events are increased in all the mutants, and the two *pri1* mutations seem to have a greater effect on gene conversion. We have not ruled out the possibility that this preferential effect on gene conversion is allele-specific rather than being uniquely characteristic of *pri1* mutations. Moreover, the meaning of such differential effect seen with other hyperrecombination mutations is not yet understood (AGUILERA and KLEIN 1988). The fact that *pri1* and *pri2* mutations increase recombination rate by only about one order of magnitude suggests that neither of the primase subunits plays a major role in mitotic recombination. Rather, as suggested for other mutants affected in DNA metabolism which show a similar hyperrecombination phenotype (HARTWELL and SMITH 1985; LUCCHINI *et al.* 1990; PETES, MALONE and SYMINGTON 1991), recombination might be stimulated by accumulation of nicks and gaps in the DNA of *pri1* and *pri2* mutant strains.

The spontaneous mutation rate is also increased 4–10-fold over the wild-type rate in all the primase mutants, as well as in the four *pol1* mutant strains we tested as controls. A similar pattern has been observed for other DNA replication conditional mutants, suggesting that DNA damage caused by defects in the DNA replication apparatus is repaired by an error-prone pathway (HARTWELL and SMITH 1985). We favour this hypothesis for the *pri1* and *pri2* mutants, since there is no evidence for *PRI1* and *PRI2* being directly involved in the repair of spontaneous DNA damage. Furthermore, we could not detect any difference in UV or MMS sensitivity between wild-type and *pri1-1*, *pri1-2* or *pri2-2* strains at any temperature. The very tight ts *pri2-1* strain showed a modest 2–4-fold increase in both UV and methyl methanesulfonate (MMS) sensitivity only when incubated for 3 hr at 37° preceding UV irradiation or after plating on YPD containing 0.02% or 0.04% MMS (our unpublished observation). Therefore, the two primase subunits do not play a major role in the repair of UV- or MMS-induced DNA damage.

Both *pri1* and *pri2* mutations severely affect the efficiency of sporulation, and unsporulated mutant cells arrest with a single nucleus. Moreover, at tem-

perature conditions which allow sporulation in the mutants, the resulting tetrads consistently contain inviable spores. Such spore inviability might be explained by improper chromosome segregation. In fact, a high frequency of chromosome loss has been observed in other DNA replication mutants, such as *cdc2* and *cdc17* (HARTWELL and SMITH 1985), and has been attributed to accumulation of DNA damage resulting from imperfect DNA replication. Unfortunately, our attempts to compare the DNA content of our wild-type and mutant strains during sporulation by flow cytometry or radioactive labeling did not provide a clear pattern, due to the rather inefficient and asynchronous sporulation that occurs in our wild-type strains and to clumping and swelling of the mutant cells. Therefore, although our data strongly implicate DNA primase in premeiotic DNA synthesis, as shown for the associated polα (BUDD *et al.* 1989), the final demonstration of this point must await more detailed analyses.

From our data thus far, mutations in either one of the two primase genes lead to similar genetic defects, and all pairwise combinations of the *pri1* and *pri2* conditional alleles under study result in a lethal phenotype. These results provide further evidence for a direct interaction between the two subunits of primase *in vivo*; however, they do not provide any information about the possibility of different roles or unique interactions of the two polypeptides with each other, with polα or with other components of the DNA replication machinery. On the other hand, it is significant to know more about the nature and severity of the biochemical defect caused by a particular mutation, while analyzing its effects on various DNA transactions *in vivo*. Our biochemical characterization of the primase subunits has shown that the *pri1-1* and *pri2-1* gene products are present at low levels in yeast crude extracts from the corresponding mutants, whereas the same extracts contain normal level of wild-type p58 or p48 subunit, respectively. Immunochromatographic purification of polα from these extracts, under conditions allowing isolation of the intact polα-primase complex from wild-type extracts, shows clearly that a normal amount of wild-type p58 is associated with the polα polypeptide even when the p48 subunit is nearly absent (*pri1-1* extract), whereas the amount of wild-type p48 associated with polα was diminished in parallel with a reduction in the level of p58 (*pri2-1* extract). Therefore, p58-polα association seems to occur in the absence of p48, whereas the p48-polα interaction appears to require the presence of the large primase subunit, which either mediates or stabilizes the association between p48 and polα. On the other hand, we find approximately the same low and ts primase activity in both *pri1-1* and *pri2-1* immunoprecipitates, which contain approximately the same amount of mutated or wild-type p48, respectively, but either a normal amount of wild-type p58 (*pri1-1*) or almost none of the mutated polypeptide (*pri2-1*). Therefore, the level of primase activity correlates with the amount of p48, independently on the presence of p58, suggesting that the small subunit plays a major role in determining enzyme activity. In this case, the temperature-sensitivity observed in the *pri2-1* immunoprecipitate, that contains wild-type p48, might be ascribed to an increased thermolability of the p48 protein in the absence of p58. These assumptions are supported by our recent demonstration that isolated wild-type p48 is sufficient for RNA primer synthesis *in vitro*, and it is more heat-sensitive than when associated with p58 (SANTOCANALE *et al.* 1993).

Further biochemical characterization of the wild-type and mutant primase subunits, as well as the search for unlinked genetic suppressors of the *pri1* and *pri2* conditional mutations, are in progress to confirm the proposed different roles of the two subunits in enzyme activity and to better understand their interactions within the polα-primase complex and with other components of the DNA replication machinery.

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