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Stable Denaturation of Chromosomal DNA from *Saccharomyces cerevisiae* During Meiosis

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Partial denaturation of *Saccharomyces cerevisiae* chromosomal DNA was found to occur spontaneously during meiosis. Short regions of strand separation (300 base pairs long) were seen in DNA molecules prepared for electron microscopy by the aqueous spreading technique. These regions were clustered along the DNA. The time course of their appearance indicated that the denatured regions were present during the periods of premeiotic DNA replication and recombination. A similar pattern of denaturation was also detected in the DNA from vegetatively grown cells of a conditional *cdc8* mutant, which is defective in DNA replication.

Intermediates of genetic recombination have been observed in the DNA molecules of bacteriophages T4 (4) and λ (31) and colicin E1 (25). The potential for detecting similar intermediates in the chromosomal DNA of eucaryotes is probably limited, however, by the greater sizes of these molecules (16) as well as by the relatively lower frequency of crossovers along their length. Among eucaryotes, *Saccharomyces cerevisiae* should be an advantageous organism in which to search for intermediates of recombination, because the diploid genome consists only of about 2×10^4 kilobases (kb) of DNA (each kb equaling 1,000 base pairs), so that chromosomal DNA molecules can be freed from the cells without breakage and can be wholly visualized by electron microscopy (22). Moreover, the genome of *S. cerevisiae* is well characterized and is known to experience about 70 crossovers per meiosis (20). Consequently, any topological structures involved in crossing over should be spaced closely enough along the homologous DNA molecules (100 to 150 kb apart) to permit their frequent detection by electron microscopy. Simchen and Friedmann (29) have found that meiotic yeast DNA isolated on CsCl gradients contained paired projections of single-stranded DNA (ssDNA) attached by one end to the duplex molecule. In our study, we have found that meiotic DNA recovered from sucrose gradients contains clusters of small denatured regions, which are described in this report.

MATERIALS AND METHODS

Cell growth. *S. cerevisiae* strain X322, provided by G. Simchen, was grown at 30°C in PSP2 medium (27) supplemented with (per liter) 40 mg of adenine and 10 mCi of [6-^{3}H]uracil (New England Nuclear). After 40 h of continuous exponential growth to a final density of 1.0×10^7 cells per ml, cells were collected by filtration, resuspended at the same cell density in sporulation medium (SPM) (27), and incubated further at 30°C.

Strain 198D1, a diploid homozygous for the allele *cdc8-1* (9), was provided by L. H. Hartwell. Cells were grown to mid-log phase in Y-minimal medium (8) supplemented with (per liter) 50 mg of adenine, 5 mg of uracil, 5 mg of histidine, 40 mg of tyrosine, 40 mg of lysine, 0.05% yeast extract (Difco), and 10 mCi of [6-^{3}H]uracil.

DNA preparation. Spheroplasts were rapidly prepared by a modified pretreatment method (21). Cells were washed twice with water by membrane filtration (Millipore) and incubated 5 min at the growth temperature in 0.1 M β-mercaptoethanol-0.2 M tris(hydroxymethyl)aminomethane, pH 9.0-0.02 M ethylenediaminetetraacetic acid (EDTA) and transferred to 1 M sorbitol-0.02 M sodium citrate-phosphate buffer (pH 5.9)-0.01 M EDTA before addition of 0.1 volume of glusulase (Endo Laboratories). Incubation at the growth temperature yielded spheroplasts in about 10 min. A portion of the spheroplast suspension was gently layered with a large-bore pipette over a 0.1-ml layer of 5% Sarkosyl in 1 M sorbitol-25 mM sodium citrate-phosphate buffer-10 mM EDTA (pH 5.9) on top of a 4.8-ml linear 15 to 30% sucrose gradient containing 1 M NaCl-10 mM EDTA-10 mM tris(hydroxymethyl)aminomethane-hydrochloride (pH 8.0). The gradients were incubated at room temperature for 5 to 10 min to permit lysis. The gradients were then run at 5°C in a Spinco 50L or 50.1 rotor at 35,000 rpm for 3.5 h. Under these conditions, there is a slight rotor speed effect, causing all DNA molecules larger than T4 DNA to be concentrated in a single narrow zone. After centrifugation, the gradients were fractionated slowly to avoid shear (22).

Fractions from sucrose gradient sedimentations were added to saturated CsCl for isopycnic banding;

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[^{14}C]DNA from a ρ° derivative (lacking mitochondrial DNA) of yeast strain A364A D-5 was added as a density marker. After adjusting the solution to a density of 1.690 g cm^{-3} with water, ultracentrifugation was performed in the Spinco 50 Ti rotor at 33,000 rpm for 60 h. Fractions were collected and analyzed for radiolabeled DNA by precipitation and scintillation counting (23).

Electron microscopy. DNA was prepared for electron microscopy by the aqueous spreading technique described by Davis et al. (5) as modified by Petes and colleagues (22). Grids were examined in a Philips EM300 electron microscope.

DNA synthesis. DNA synthesis was followed by measuring the increase in total cellular DNA content by a modification (10) of the diaminobenzoic acid flourometric procedure of Kissane and Robins (17).

Nuclear staining. Cells were prepared for the light microscopic determination of nuclear stages by a modification (8) of the Giemsa staining methods of Robinow and Marak (26).

Intragenic recombination. The stimulation of intragenic recombination in a *cdc8-1* homozygote heteroallelic at the *ade2* locus during temperature-sensitive arrest of vegetative growth was assayed by plating portions on plates lacking adenine. Portions plated on complete nutrient plates served to assay the plating efficiency under nonselective conditions.

RESULTS

Isolation of DNA. Standard spheroplasting techniques (23) failed to yield DNA as large as that obtained from vegetative cells, possibly because deoxyribonucleases are extremely active in these cells (15) and because the walls of sporulating cells are particularly refractory to removal by glusulase. Therefore, the modified spheroplasting technique described in the text was adapted for isolation of DNA. Figure 1 shows a sucrose gradient sedimentation profile of DNA released from cells after 6 h in SPM. A large peak of DNA molecules greater in size than the T4 DNA marker was obtained under these conditions.

Fractions of the gradient were pooled as indicated in Fig. 1 for isopycnic banding in cesium chloride gradients. The profiles, shown in Fig. 2, revealed that the DNA in the high-molecular-weight peak was of nuclear density, whereas both nuclear and mitochondrial DNAs were present in the peak of DNA smaller than the T4 marker.

Electron microscopy of meiotic DNA. The topology of chromosomal DNA during meiosis was determined by electron microscopy of the molecules in the high-molecular-weight peak. To avoid shear, the peak was recovered from an identical sucrose gradient run in parallel with the analytical gradient but fractionated through a large-bore needle (13 gauge). Samples of DNA from cells at 3, 4, 6, 8, and 10 h in SPM were prepared for electron microscopy by the aqueous protein monolayer technique and examined in the electron microscope.

The types of molecules recovered from cells at each stage of meiosis are listed in Table 1.

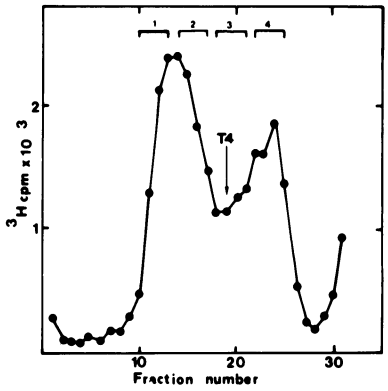

FIG. 1. *Sedimentation pattern of DNA from a sporulating culture. DNA was prepared from X322 cells after 6 h of incubation in SPM. The direction of sedimentation is from right to left. Fractions of an identical gradient run in parallel were pooled for cesium chloride analysis as indicated. Marker T4 DNA sedimented in fraction 19 under identical conditions in another gradient.*

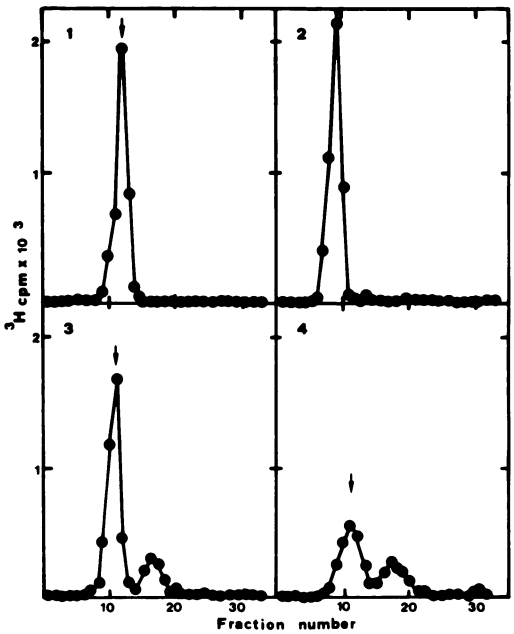

FIG. 2. *Isopycnic banding of DNA from cells incubated for 6 h in SPM. Pooled fractions from sucrose sedimentation of X322 cells, shown in Fig. 1, were analyzed by cesium chloride centrifugation. The arrow indicates the density equilibrium position of a ^{14}C-labeled ρ° marker in each gradient.*

TABLE 1. *Types of DNA molecules from sporulating cells*

Sample (h in SPM)	No. of molecules examined	Linear		Denatured regions[a]		Replication intermediates	
		No.	%	No.	%	No.	%
3	53	53	100	0	0	0	0
4	51	49	96	0	0	2	4
6[b]	102	92	90	7	7	3	3
	71	64	90	7	10	0	0
	122	111	91	10	8	1	1
8	85	77	91	7	8	1	1
10	52	51	98	0	0	1	2

[a] Number and percentage of molecules containing one or more denatured regions.
[b] Three independent experiments.

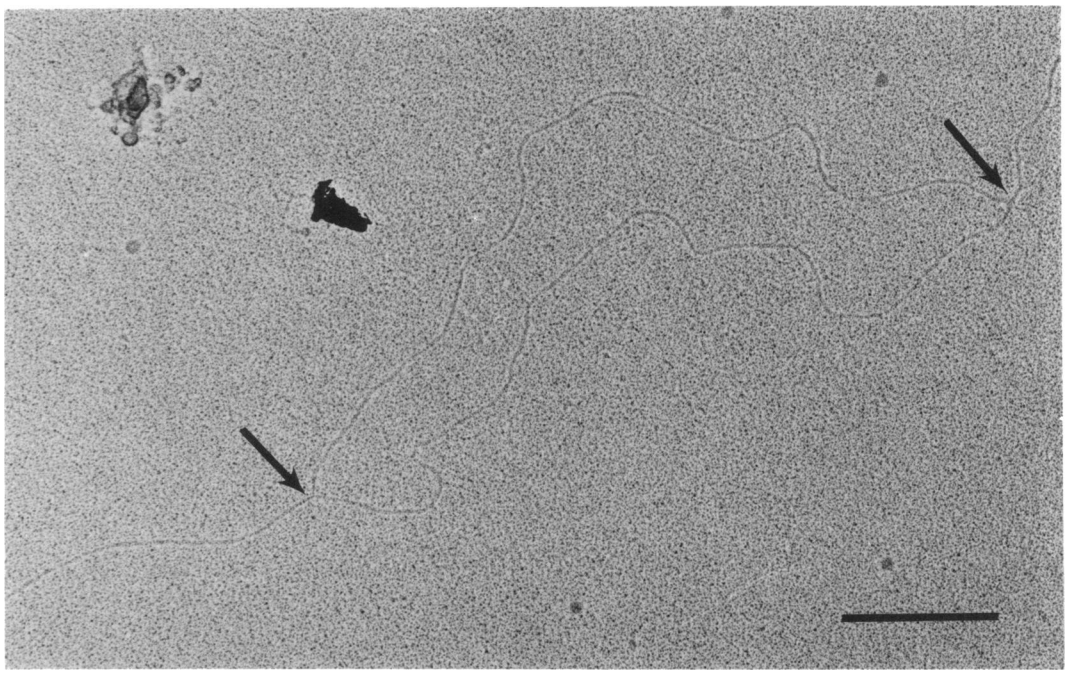

FIG. 3. *Electron micrograph of a 6-kb-long DNA replication bubble (between arrows) from a sporulating culture. The sample was prepared from a sucrose gradient of DNA isolated from X322 cells incubated for 6 h in SPM. The bar represents 1 kb.*

Most of the molecules observed were linear. A few were interpretable as replication structures, including internal bubbles and terminal forks. A typical bubble isolated from cells at 6 h in SPM is shown in Fig. 3.

These preparations also revealed molecules with another structural alteration. Molecules isolated from cells at 6 and 8 h in SPM frequently contained short regions of strand separation. These regions differed from replication bubbles in that both strands were thinner and of more irregular contour than the interstitial DNA segments, suggesting that they represent regions of denaturation (Fig. 4). These regions were approximately 300 bases (0.3 kb) in length and occurred in clusters with interstitial duplex segments 1.5 to 3.0 kb in length. This clustering is illustrated by the molecule traced in Fig. 5. This molecule (180 kb in length) contains a central cluster of 30 denatured regions within a 69-kb segment. The two ends of the molecule also contain a few denatured regions, but the clusters are divided from one another by long segments devoid of any apparent denaturation.

Correlation of denaturation with stages of meiosis. The occurrence of the small denatured regions in meiotic DNA was limited to a restricted period of the meiotic process. Table 1

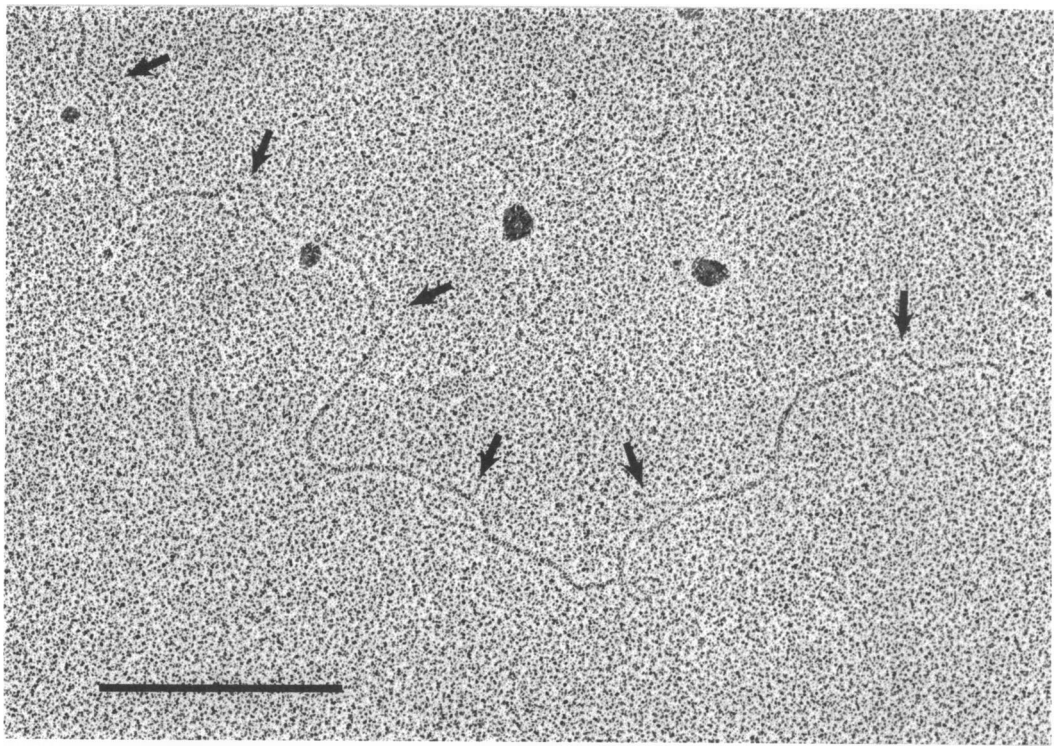

FIG. 4. *Electron micrograph of a cluster of denatured regions. A cluster of six denatured regions (indicated by arrows) is shown in a molecule from X322 cells incubated for 6 h in SPM. The bar represents 1 kb.*

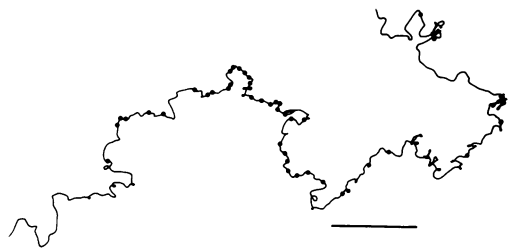

FIG. 5. *Tracing of an electron micrograph of a DNA molecule containing denatured regions. The sample was prepared from a sucrose gradient sedimentation of DNA from X322 cells incubated for 6 h in SPM. The small circles designate the position of the denatured regions. The bar represents 10 kb.*

shows that 7% of the molecules from cells at 6 and 8 h in SPM contained denatured regions. The high degree of reproducibility of this result is demonstrated by the data (6-h entries) from three separate experiments. Molecules taken from cells at the indicated earlier and later stages were devoid of denatured regions.

To determine which meiotic events were coincident with the occurrence of denaturation, premeiotic DNA replication and meiotic division were monitored. The kinetics of meiosis I and meiosis II were determined by Giemsa staining of the nuclei. Although meiosis I does not result in complete division of the nucleus in this organism (19), transitions to apparent binucleate and tetranucleate stages provide indicators of the two meiotic divisions. The kinetics of the appearance of binucleate and tetranucleate cells (Fig. 6A) indicate that meiosis I and meiosis II began at about 6 and 8 h, respectively. Premeiotic DNA replication, assayed by the diaminobenzoic acid fluorometric method (10), began at 2 h in SPM and was completed by 10 h (Fig. 6b). The observed asynchrony of the meiotic divisions implies, however, that a major portion of the replication period for the entire culture results from asynchrony between the replication periods for individual cells. Assuming that the S phase for all cells is of equal extent but asynchronous in its occurrence, we can derive its extent by determining the asynchrony of entry into a subsequent event, meiosis I. We find that the total proportion of cells which have entered meiosis I (the sum of binucleates and tetranucleates in Fig. 6b) rises from 5% to 95% of the plateau value between 5.5 and 9 h in SPM, whereas the increase in DNA content rises over similar limits between 2 and 9 h in SPM. There-

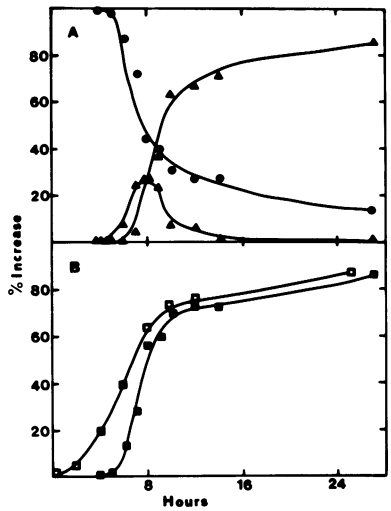

FIG. 6. *Kinetics of nuclear division during meiosis. After vegetative growth in PSP2 medium, a culture of strain X322 was transferred to SPM. At the indicated times, samples were removed for analysis of DNA content and nuclear morphology. (A) Determination of nuclear division by Giemsa staining. ●, Mononucleate cells; △, binucleate cells; ▲, tetranucleate cells and asci. (B) DNA replication and completion of meiosis I. □, Increase in cellular DNA content; ■, completion of meiosis I, taken as the sum of binucleate and tetranucleate cells, as given in (A), at each time point.*

fore, the total extent of DNA replication (7 h) is 3.5 h longer than that of entry into meiosis I, implying that asynchrony accounts for 3.5 h of the overall replication period. This extent of asynchrony approximates the expected time for cells to complete vegetative cycles upon transfer from PSP2 to SPM. The remaining 3.5 h may be taken as the extent of the S phase for each individual cell.

On the basis of this derivation, we may determine which phases of meiosis could be coincident with the observed denaturation of chromosomal DNA. At 4 h in SPM, one-fourth of the aggregate DNA replication had occurred (Fig. 6B), but no denaturation was evident (Table 1). Therefore, no denaturation accompanied the early portion of DNA replication occurring in a substantial fraction of cells. Moreover, denaturation must not have occurred during meiosis I, because there was no increase in the frequency of denatured regions between 6 and 8 h in SPM, while there was a threefold increase in the frequency of binucleate cells. The denatured regions must therefore be present after the early portion of the S phase but before meiosis I. The data do not permit a distinction between their occurrence during the latter part of S phase or during ensuing events of meiotic prophase.

Electron microscopy of DNA from vegetative cultures of a *cdc8* strain. During the course of these studies, small denatured regions were also detected in DNA isolated from temperature-arrested vegetative cultures of strain 198D1, which is homozygous for a mutation in the *cdc8* gene. When 198D1 cells grown in vegetative medium (YM-1) are shifted from 23 to 36°C, they fail to undergo DNA replication and become arrested at the stage of medial nuclear division (9). DNA prepared from cells 2 h after the shift to the restrictive temperature was examined by electron microscopy and found to contain clusters of small denatured regions. As in the case of meiotic DNA, the denatured regions in DNA from *cdc8* cells were 300 bases in length and were clustered with duplex interstitial regions of 1.5 to 3.0 kb. On the other hand, electron microscopic examination of DNA from 198D1 cells grown only at the permissive temperature did not reveal any molecules with denatured regions (Table 2).

Denatured regions were present in 21% of the molecules examined from temperature-arrested 198D1 (Table 2). The higher frequency of molecules with denatured regions in this case than in the meiotic cells facilitated testing the sensitivity of these regions to various agents. When the DNA recovered from sucrose gradients was subjected to equilibrium banding on a CsCl gradient, the occurrence of denatured regions was reduced by more than 90%. In addition, a treatment of gradient-isolated DNA with proteinase K reduced the frequency of molecules with denatured regions from 21% to less than 10% (9 of 95 molecules) in one experiment; further treatment was not attempted. These results suggest that the DNA is maintained in a single-stranded configuration by an electrostatic interaction with protein.

Since the *cdc8* strain is temperature sensitive for the propagation of DNA synthesis, replicative intermediates also accumulate in the DNA

TABLE 2. *Types of DNA molecules from cdc8 cells*

Sample	Linear		Replication intermediates		Denatured regions[a]	
	No.	%	No.	%	No.	%
198D1 (room temperature)	93	90	10	10	0	0
198D1 (36°C, 2h[b])	87	64	26	19	29	21

[a] Number and percentage of molecules containing one or more denatured regions.

[b] The sum of the frequencies exceeds 100% because molecules containing both replication intermediates and denatured regions are included in both columns.

of cells held at 36°C (24). These replicative intermediates are distinguishable from the denatured regions by their being longer as well as by their having the apparent thickness of duplex DNA. To investigate the relationship between the denatured regions and these replicative intermediates, the frequency with which each appeared on the DNA molecules was determined. Whereas 19% of the molecules contained replicative intermediates and 21% contained denatured regions, only 4% contained both together. Since the random coincidence of the two would also be 4%, there is no significant correlation between the occurrence of the two types of structures. This conclusion is confirmed by the observation of denatured regions both on replicated DNA (on replication bubbles) and on unreplicated segments (between replication bubbles).

Temperature stimulation of mitotic intragenic recombination. Because of the possibility that similar denatured regions in DNA from sporulating cells play a role in recombination, the effect of incubation at the restrictive temperature on intragenic recombination in 198D1 was examined. Mitotic intragenic recombination at the *ade2* locus was stimulated to six times the basal rate of adenine prototroph formation by a shift to the restrictive temperature, whereas no increase in prototroph formation was observed in the homoallelic control. This level of stimulation is locus specific, since intragenic recombination is stimulated 13-fold at the *leu-1* locus, but only 1.6-fold at the *arg4* locus (F. Fabre, personal communication). By comparison, the intragenic recombination at the *ade2* locus could be stimulated 80-fold by UV irradiation of stationary-phase cells.

DISCUSSION

In the present study, we found that the single-stranded regions are most frequently present as short segments of strand separation within a principally duplex molecule. Structures of similar appearance have been seen in a variety of cases, including superhelical DNA of phage PM2 (3, 14), D-loops of replicating λ DNA (13), "R-loops" of T7 DNA during transcription in vitro (2), and rapidly replicating DNA of *Drosophila* embryos (33). In none but the present case has strand separation been demonstrated under aqueous conditions, which should permit unmodified DNA to collapse into "bushes" or undergo rapid renaturation (5). The possible modification of yeast DNA suggested by the stability of strand separation has been examined in the molecules isolated from vegetatively arrested *cdc8* cells, where such regions occur at high frequency. The disappearance of these regions upon centrifugation in CsCl gradients or digestion with proteinase K suggests that the DNA is modified by the noncovalent binding of a protein. A DNA-binding protein has, in fact, recently been isolated from DNA molecules bearing the denatured regions (manuscript in preparation). The presence of a DNA-binding protein here might appear inconsistent with the fact that the gene 32 product of phage T4 causes DNA to appear thicker (7). Recent observations on the in vitro binding of the adenovirus 2 DNA-binding protein demonstrate, however, that thickening is seen only after treatment of the sample with a fixative (glutaraldehyde) before spreading (H. L. Klein, unpublished data).

(ssDNA) is known to be present during both replication and recombination. Electron microscopy of replication intermediates from phages λ (12), T7 (32), and T4 (6) and from *Drosophila* embryos (18) has demonstrated extensive gaps of ssDNA in daughter strands as well as single-stranded "whiskers" extending outward from replication forks. Segments of ssDNA have also been found at the sites of cross-strand exchange in recombinational intermediates of T4 DNA (4) and λ DNA (31). Although the precise function of ssDNA is obscure, single strandedness is clearly essential in at least one instance: a functional DNA-binding protein encoded by phage T4 gene 32 is necessary for both replication and recombination in T4-infected bacteria (1). No essential function has been similarly demonstrated in meiosis, but a DNA-binding protein has been found to become associated with *Lilium* DNA during meiotic prophase (11). The occurrence of denatured regions in yeast, as described in this report, indicates a role for ssDNA during some phase of meiosis here as well. The strain under study carried no heteralleles useful for assaying the stimulation of intragenic recombination (28), but a closely related strain (AP-1) has been shown to undergo stimulation of conversion to prototrophy at the *ade2* locus during the earlier part of the DNA replication period (10). The critical events of recombination may, however, not occur until sometime after the stimulation (10). The kinetics of the denaturation itself (Table 1) indicate that this event occurs during a phase which follows the majority of replication.

The present report also describes similar clustered segments of strand separation during the temperature-sensitive arrest of vegetative growth in a *cdc8* mutant. Although the fact that this mutant is specifically defective in the propagation of DNA replication (9) might indicate that denatured regions represent accumulated replicative intermediates, we find that they oc-

cur both on replicated and unreplicated strands. Any role of the initiation of replication would therefore only be consistent with the anomalous reinitiation of replication on daughter strands. Unrau and Holliday (30) have described a similar mutant of *Ustilago maydis* that is also temperature sensitive for DNA synthesis. Here, a stimulation of mitotic recombination occurs during the inhibition of DNA synthesis. It would be of interest to examine the DNA from this mutant to see if it also contains denatured regions.

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