MOLECULAR AND CELLULAR BIOLOGY, Aug. 1991, p. 4111–4120 Vol. 11, No. 8
0270-7306/91/084111-10$02.00/0
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The Highly Conserved N-Terminal Domains of Histones H3 and H4 Are Required for Normal Cell Cycle Progression

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Received 1 March 1991/Accepted 28 May 1991

The N-terminal domains of the histones H3 and H4 are highly conserved throughout evolution. Mutant alleles deleted for these N-terminal domains were constructed in vitro and examined for function in vivo in *Saccharomyces cerevisiae*. Cells containing a single deletion allele of either histone H3 or histone H4 were viable. Deletion of the N-terminal domain of histone H4 caused cells to become sterile and temperature sensitive for growth. The normal cell cycle progression of these cells was also altered, as revealed by a major delay in progression through the G_2+M periods. Deletion of the N-terminal domain of histone H3 had only minor effects on mating and the temperature-sensitive growth of mutant cells. However, like the H4 mutant, the H3 mutants had a significant delay in completing the G_2+M periods of the division cycle. Double mutants containing N-terminal domain deletions of both histone H3 and histone H4 were inviable. The phenotypes of cells subject to this synthetic lethality suggest that the N-terminal domains are required for functions essential throughout the cell division cycle and provide genetic evidence that histones are randomly distributed during chromosome replication.

The nucleosome consists of the four core histones H2A, H2B, H3, and H4 arranged as two H2A-H2B dimers and one H3-H4 tetramer, around which is wrapped approximately 146 bp of DNA. Each histone is composed of a main globular domain of high α-helical content and an extended N-terminal segment of 12 to 26 amino acids. The globular regions of the histones contain roughly equal distributions of acidic and basic amino acids, and it is these globular domains that participate in the major histone-histone and histone-DNA interactions that maintain the integrity of the core particle (38). The N-terminal domains are distinct in amino acid composition and sequence from the rest of the protein and contain a high concentration of positively charged residues (13). Digestion of isolated chromatin or nucleosomes with trypsin or chymotrypsin results in the specific proteolysis of the N-terminal domains leaving a reproducible set of protected fragments (Fig. 1) (5, 26, 41). Many of the posttranslational modifications of the histones, including acetylation, methylation, and phosphorylation, are also largely confined to amino acids in the N-terminal domains (Fig. 1) (7, 19, 44).

The N-terminal domains probably do not play a major role in maintaining core particle structure. Trypsinized core particles show little structural perturbation, and histone octamers in which the N-terminal domains have been removed are capable of folding DNA into reconstituted core particles (18, 42, 43). Nevertheless, at least part of the N-terminal regions may interact with the DNA and further stabilize nucleosome structure. For example, the N-terminal region of histone H4 can be physically cross-linked to nucleosomal DNA in vitro (10). Trypsinized core particles show specific alterations in the accessibility of the DNA to DNase I digestion (2, 42), and both trypsinization and hyperacetylation of the lysines in the N-terminal domains appear to destabilize the nucleosome to thermal melting and torsional stress (2, 24). The N-terminal domains have also been shown to be required for the formation of higher-order chromatin structure in vitro (1).

Genetic studies in *Saccharomyces cerevisiae* have shown that the N-terminal basic domains of either H2A or H2B can be deleted without loss of viability. The N-terminal domains can even be exchanged between the proteins with little effect; however, the deletion of both domains is lethal (28, 39). These results imply that the individual N-terminal regions of H2A and H2B are not required for nucleosome formation but do complement each other for one or more unknown essential functions. As shown by Kayne et al. (16) and the work reported here, the N-terminal domain of histone H4 can also be deleted without loss of viability; however, this deletion results in a number of phenotypic defects.

Several functional roles have been proposed for the histone N-terminal regions. A specific set of amino acids in the N-terminal domain of histone H4 is required for the permanent inactivation of the yeast silent mating type loci *HML* and *HMR* (15, 16, 20, 25). The four lysine residues subject to reversible acetylation in the N-terminal domain of histone H4 have been found to be important for several cellular functions, including DNA replication and chromosome segregation (20). To date very little is known about the N-terminal domain of histone H3, although, like histone H4, it has maintained a high degree of conservation throughout evolution. Finally, acetylation of the histone H3 and H4 N-terminal domains affects the superhelical density of circular plasmid DNA reconstituted into chromatin in vitro, suggesting that the nucleosome may act as a eukaryotic DNA gyrase (23).

In this report we describe the construction and characterization of deletion mutations of the N-terminal domains of histones H3 and H4 in *S. cerevisiae*. We sought to determine the roles of these domains in the expression of the silent mating type loci, cell division cycle progression, and general viability and growth characteristics. Double mutants were examined to determine whether these domains share common functions and how these functions influence the cell cycle, growth, and inheritance.

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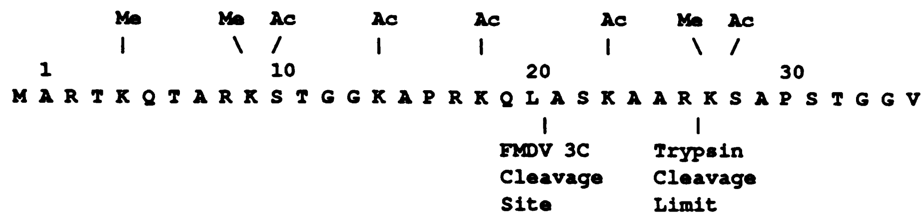
N-Terminus of H3



N-Terminus of H4

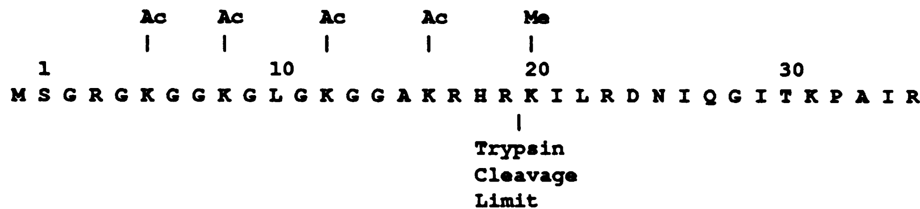


FIG. 1. The first 35 amino acids of histones H3 and H4 in *S. cerevisiae*. The potential acetylation (Ac) and methylation (Me) sites are indicated (19, 44). The limits of trypsin digestion (5) and the cleavage site of the FMDV 3C protease (11) are also shown.

MATERIALS AND METHODS

Bacterial and yeast strains. The bacterial strains were all derivatives of *Escherichia coli* K-12. Strain JM83 [*ara* Δ(*lac pro*) *thi lacZ*Δ*M15*] (21, 45), ED8654 (*supE supF* r^- m^+ *met trpR*) (22), or JA221 (r^- m^+ *lacY leuB6* Δ*trpE5 recA1*) (8) was used for bacterial transformations.

The *S. cerevisiae* strains used in the plasmid shuffle protocol were MX4-22A [*MAT***a** *ura3-52 leu2-3,112 lys2*Δ*201* Δ(*HHT1-HHF1*) Δ(*HHT2-HHF2*) pMS329] (20) and MX1-4C [*MAT*α *ura3-52 leu2-3,112 trp1 his3* Δ(*HHT1-HHF1*) Δ(*HHT2-HHF2*) pMS329] (this work). The strains used for the mating test were MSY108 (*MAT*α *his1*) (20) and MSY159 (*MAT***a** *ura3-52 ade2-101 lys2-801*) (34).

Plasmids and allele construction. The plasmid pMS329 was derived from YRp14CEN4 and contained the wild-type histone H3 and H4 genes *HHT1* and *HHF1*, as well as the yeast marker genes *URA3* and *SUP11* (20). The plasmid pMS337 was an *ARS1/CEN4* plasmid that also contained the wild-type histone H3 and H4 genes *HHT1* and *HHF1* but carried the yeast marker gene *LEU2*.

The plasmid pKT41 (kindly provided by K. B. Freeman) was used to construct the deletion alleles of histone H3 *hht1-1* and *hht1-2*. This plasmid was derived from Bluescript (Stratagene, La Jolla, Calif.) and contained the 900-bp *Cla*I-*Sma*I yeast fragment that carries the copy-I H3 gene (31–33). To construct the *hht1-1* allele, pKT41 was digested with *Cla*I and *Bal*I, releasing H3 on a 703-bp *Cla*I-*Bal*I fragment. This fragment was further digested with *Alu*I, which cuts in the codon for Ala-21 (Fig. 2), generating a 644-bp *Cla*I-*Alu*I fragment missing the codons for amino acids 1 to 20. This fragment was then cloned between the *Cla*I and *Bal*I sites of the original pKT41 vector, creating an in-frame fusion deleted for codons 1 to 20 (Fig. 2). To construct the *hht1-2* allele, we used the polymerase chain reaction (PCR) to amplify a DNA fragment of the H3 gene in pKT41 from the codon for Ala-28 to the *Cla*I site past the C terminus of H3. This was achieved by using two oligonucleotides that hybridize to the region at Ala-28 (5′-CCCCATCTACCGGTGGT GTT-3′) and the Bluescript vector at the *Cla*I site (5′-CTCGAGGTCGACGGTATCGA-3′). The 637-bp amplified DNA fragment was purified, digested with *Cla*I, and then cloned between the *Cla*I and *Bal*I sites in pKT41. This ligation resulted in an in-frame deletion of the codons for amino acids 1 to 28 (Fig. 2).

The vector used to construct the histone H4 deletion allele *hhf1-8* carried a 476-bp *Rsa*I fragment containing the copy-I histone H4 gene *HHF1* (31–33) cloned into the *Bam*HI site of M13mp8 with *Bam*HI linkers. An oligonucleotide was used to create a *Taq*I restriction site by introducing a G-to-C substitution at nucleotide 80 of the H4 coding sequence (Fig. 2). This substitution replaced Gln-27 with a glutamic acid residue. The 128-bp *Bam*HI-*Msp*I fragment and the 360-bp *Taq*I-*Bam*HI fragment were then ligated and cloned into the *Bam*HI site of M13mp8 to create an in-frame deletion of codons 2 to 26 (Fig. 2). The DNA sequences of the *hht1-1*, *hht1-2*, and *hhf1-8* alleles were obtained to confirm that the deletion end points were correct and in frame. Additionally, the entire coding region of *hht1-2* was sequenced, and no additional base changes were found.

Appropriate restriction fragments carrying each of the three alleles were used to replace either *HHT1* or *HHF1* in plasmid pMS337 to create plasmids pMS357 (*hht1-1 HHF1*), pMS358 (*hht1-2 HHF1*), and pMS338 (*HHT1 hhf1-8*). The plasmid pMS347 contains only the copy-I histone H3 gene *HHT1* and was derived from pMS337 by deleting the *Bam*HI fragment carrying the H4 gene. Finally, the *HHF1* genes in plasmids pMS357 and pMS358 were replaced with the *hhf1-8* allele to create plasmids pMS359 (*hht1-1 hhf1-8*) and pMS360 (*hht1-2 hhf1-8*).

To construct the plasmid pMS362, used for conditional expression of histone H4, an *Eco*RI-*Bam*HI restriction fragment containing the *GAL1* promoter from the plasmid

A. Construction of *hht1-1*

1 10 20 30
M A R T K Q T A R K S T G G K A P R K Q L A S K A A R K S A P S T G G V
ATGGCCAGAACAAAGCAAACAGCAAGAAAGTCCACTGGTGGTAAGGCCCCAAGAAAGCAATTAGCTTCTAAGGCTGCCAGGAAATCCGCCCCATCTACCGGTGGTGTT
TACCGGTCTTGTTTCGTTTGTCGTTCTTTCAGGTGACCACCATTCCGGGGTTCTTTCGTTAATCGAAGATTCCGACGGTCCTTTAGGCGGGGTAGATGGCCACCACAA
BalI AluI

22 30 35
M A S K A A R K S A P S T G G V
ATGGCTTCTAAGGCTGCCAGGAAATCCGCCCCATCTACCGGTGGTGTT
TACCGAAGATTCCGACGGTCCTTTAGGCGGGGTAGATGGCCACCACAA

B. Construction of *hht1-2*

1 10 20 30
M A R T K Q T A R K S T G G K A P R K Q L A S K A A R K S A P S T G G V
ATGGCCAGAACAAAGCAAACAGCAAGAAAGTCCACTGGTGGTAAGGCCCCAAGAAAGCAATTAGCTTCTAAGGCTGCCAGGAAATCCGCCCCATCTACCGGTGGTGTT
TACCGGTCTTGTTTCGTTTGTCGTTCTTTCAGGTGACCACCATTCCGGGGTTCTTTCGTTAATCGAAGATTCCGACGGTCCTTTAGGCGGGGTAGATGGCCACCACAA
BalI PCR

30 35
M A P S T G G V
ATGGCCCCATCTACCGGTGGTGTT
TACCGGGGTAGATGGCCACCACAA

C. Construction of *hhf1-8*

1 10 20 (Q) 30
M S G R G K G G K G L G K G G A K R H R K I L R D N I E G I T K P A I R
ATGTCCGGTAGAGGTAAAGGTGGTAAAGGTCTAGGTAAAGGTGGTGCCAAGCGTCACAGAAAGATTCTAAGAGATAACATCgAAGGTATTACTAAGCCAGCTATCAGA
TACAGGCCATCTCCATTTCCACCATTTCCAGATCCATTTCCACCACGGTTCGCAGTGTCTTTCTAAGATTCTCTATTGTAGcTTCCATAATGATTCGGTCGATAGTCT
MspI TaqI

30 35
M S E G I T K P A I R
ATGTCCGAAGGTATTACTAAGCCAGCTATCAGA
TACAGGCTTCCATAATGATTCGGTCGATAGTCT

FIG. 2. Deletion construction strategy. (A) Construction of the *hht1-1* allele. The blunt-ended *Bal*I restriction site in the Ala-1 codon was ligated to the blunt-ended *Alu*I restriction site in the Ala-21 codon to create an in-frame deletion of the codons for amino acids 1 to 20 of H3. (B) Construction of the *hht1-2* allele. The *Bal*I restriction site in the Ala-1 codon was ligated to the blunt-ended PCR product (see Materials and Methods), whose 5′ end lies in the codon for Ala-28 (indicated by PCR), creating an in-frame deletion of the codons for amino acids 1 to 28 of H3. (C) Construction of the *hhf1-8* allele. An oligonucleotide was used to introduce a G-to-C substitution in the glutamine codon (Q) at position 27. This creates a *Taq*I restriction site and substitutes glutamic acid (E) for the wild-type glutamine (Q). Ligation of the *Msp*I restriction site in the codon for Ser-1 to the new *Taq*I restriction site resulted in an in-frame deletion of the codons for amino acids 2 to 26 of H4. The Q-to-E substitution is also maintained in the deletion construct.

pBM125 (kindly provided by Mark Johnston) was fused to the *Bam*HI restriction fragment containing the copy-I histone H4 gene *HHF1* from M13mp8 (described above) and a 291-bp *Bam*HI-*Pst*I fragment containing the copy-I histone *H4ARS* element. The *H4ARS* fragment was constructed by subcloning the 283-bp *Rsa*I fragment spanning the *H4*ARS (6, 32) into the *Hinc*II site of M13mp8, converting it to a 291-bp *Bam*HI-*Pst*I fragment. The *GAL1*::*HHF1*::*H4ARS* fusion was cloned between the *Eco*RI and *Pst*I sites of the *TRP1*/*CEN6*/*H4ARS* vector pRS314 to create plasmid pMS362 (30).

Reagents and DNA sequencing. Restriction enzymes were obtained from New England BioLabs (Beverly, Mass.). DNA sequencing was by the dideoxy method described previously (3, 27) with Sequenase enzyme supplied by United States Biochemical Corporation (Cleveland, Ohio). Double-strand DNA sequencing of plasmids was performed with Sequenase enzyme as described previously (37).

PCR. PCR was performed with the GeneAmp DNA Amplification Reagent Kit, including the AmpliTaq recombinant *Taq* DNA polymerase and a DNA Thermal Cycler—all supplied by Perkin Elmer Cetus (Norwalk, Conn.). To obtain the 637-bp DNA fragment spanning Ala-28 to the *Cla*I site downstream of H3, we ran 24 cycles of 94°C for 1 min, 50°C for 2 min, and 72°C for 3 min using the oligonucleotides described above. This was followed by one cycle of 94°C for 1 min, 50°C for 2 min, and 72°C for 7 min. The PCR material was extracted with phenol-$CHCl_3$, ethanol precipitated, and analyzed on a 1% agarose gel. The PCR-amplified product was isolated and used to construct *hht1-2* as described above.

Yeast growth media and transformations. The YPD and SD media for yeast growth have been described previously (29). Yeast transformations were by a lithium acetate method (14).

Plasmid shuffle protocol. The analysis of histone H3 and H4 mutations by the plasmid shuffle protocol was carried out as described previously (20). *S. cerevisiae* MX4-22A and MX1-4C were transformed with individual *LEU2/CEN4/ARS1* plasmids carrying the alleles of H3 and H4 to be tested. Transformed colonies were then spread onto plates containing 5-fluoroorotic acid (5-FOA), a drug that prevents growth of *URA3* cells, to select for cells that had lost the resident wild-type histone plasmid pMS329. The plasmid genotypes of resulting colonies were confirmed by Southern blot analysis. For conditional expression experiments, yeast host MX1-4C was transformed with the *GAL1*::*HHF1* fusion plasmid, pMS362, selecting for Trp^+ colonies. The test alleles of the histone H3 and H4 genes were then introduced on *LEU2/CEN4/ARS1* plasmids as described above and selected on Trp^- Leu^- plates with galactose as a carbon source. The plasmid shuffle was completed by plating cells on galactose 5-FOA medium to select for loss of the resident wild-type histone plasmid pMS329. Cells from these colonies contained a copy of the *LEU2/CEN4/ARS1* plasmid with the test histone alleles and a copy of plasmid pMS362 expressing the wild-type histone H4 gene from the *GAL1* promoter. Expression of the wild-type histone H4 fusion gene could then be turned off by growing cells on glucose as a carbon source.

Generation times and DNA content analyses. Generation times were calculated from cell counts obtained from exponentially growing cultures at timed intervals using a Coulter Counter model ZM particle counter (34). The DNA content of cells rescued by the different alleles was obtained essentially as described by Corliss and White (9). Cells from exponential cultures in YPD at 28°C were fixed in 70% ethanol, sonicated briefly, and treated with RNase at 30°C. Cells were then incubated with pepsin (5 mg/ml in 55 mM HCl) and stained with 2 ml of a 50-μg/ml propidium iodide solution. The DNA fluorescence histogram was collected by using an EPICS fluorescence-activated cell sorter (Coulter Electronics, Inc.). The propidium iodide was excited with a 1-W laser source at 488 nm.

Quantitative mating. For each strain to be tested, about 5 × 10^7 cells from early-log-phase cultures were mixed with an equal number of the appropriate tester strain, either MSY108 or MSY159. The cell mixture was incubated on YPD plates at 28°C for 4 h. Cells were then washed off the plates, serially diluted, and plated on appropriate selective plates. Following incubation at 28°C, the plates were scored for diploids and each haploid parent. The mating efficiency was expressed as the number of diploids as a percentage of the least frequent haploid parent.

RESULTS

Deletions of the N-terminal domains. To facilitate a genetic analysis of the N-terminal domains of histones H3 and H4, a set of deletion mutations was made as described in Materials and Methods. Two alleles of histone H3 were constructed. The first deletion, *hht1-1* (Δ1–20), was designed to code for a truncated histone H3 corresponding to the proteolytic product produced by the action of the foot-and-mouth disease virus (FMDV) 3C protease (11). The second deletion, *hht1-2* (Δ1–28), was constructed to remove the trypsin-sensitive N-terminal domain and the lysine residues subject to reversible acetylation.

The N-terminal domain deletion allele of H4, *hhf1-8* (Δ2–26), is the counterpart of *hht1-2*, as it was designed to remove the trypsin-sensitive N-terminal domain and the four sites of lysine acetylation in histone H4. The glutamine codon at position 27 was also altered to a glutamic acid codon as a consequence of the strategy used to construct the H4 deletion allele (see Materials and Methods). When this single Gln-27-to-Glu-27 mutation was examined in *S. cerevisiae*, it was found to be wild type for all phenotypes tested.

Viability of deletion mutants. The ability of each deletion allele to provide functional histone protein for cell growth was tested by using a plasmid shuffle protocol described previously (4, 20; see Materials and Methods). The advantage of this technique is that it preserves an isogenic strain background in which to test each allele.

When tested in this assay, all three N-terminal deletion alleles of histones H3 and H4 were able to support the growth of yeast colonies in two different strain backgrounds, MX4-22A and MX1-4C. DNA was prepared from several colonies of each strain after plasmid shuffle and tested by Southern blot analysis to ensure that the plasmids carried the expected deletion allele and not a recombination product between the two plasmids. The results confirmed that in each case the deletion allele was present and was the sole source of histone H3 or H4 protein for the cells. Thus, the individual N-terminal domains of histones H3 and H4 are dispensable for cell viability in *S. cerevisiae*.

Growth of mutants. The generation and cell cycle times of strains derived from MX4-22A by plasmid shuffle are summarized in Table 1. The cell cycle times were determined from the histograms of DNA content obtained by flow microfluorometry (34). All three mutant strains had longer generation times than the wild-type control. In each case, the increased generation time was primarily the result of a longer G_2+M period, although a small increase in the S period was also observed. The growth rates of strains expressing either of the H3 N-terminal deletion alleles were very similar; the generation times were about 30% longer than those of the wild-type strains, and the S and G_2+M phases of the division cycle were approximately 30 and 60% longer, respectively. The cell cycle defects were more pronounced for the H4 N-terminal deletion mutant. In this case, the apparent generation time more than doubled compared with that of the wild-type strain, and the G_2+M phases of the division cycle increased from about 66 min to more than 200 min. These results are in agreement with those of Kayne et al. (16) for a series of deletions of the N terminus of H4 and with our previous studies of point mutations in the N-terminal domain of histone H4 (20).

Mating type gene expression. Previous experiments have shown that deletions or point mutations in the N-terminal domain of H4 result in derepression of the silent mating type loci (15, 16, 20, 25). Therefore, we tested the H3 mutants for

TABLE 1. Generation times and cell division cycle periods

Histone allele	Mutation[a]	Generation time[b] (min)	Cell cycle[b] (min)			n[c]
			G_1	S	G_2+M	
HHT1 HHF1	WT	123 ± 5	25 ± 2	33 ± 3	66 ± 4	5
hht1-1	Δ(A1–L20)	164 ± 5	13 ± 1	45 ± 3	107 ± 4	6
hht1-2	Δ(A1–S28)	161 ± 6	11 ± 1	44 ± 3	107 ± 5	5
hhf1-8	Δ(G2–I26), QE27	269 ± 7	25 ± 2	42 ± 3	203 ± 6	5

[a] WT, wild-type H3 and H4 alleles. The deletions are described by the first and last amino acid deleted. The substitution of the glutamine residue at position 27 of H4 to a glutamic acid residue in the *hhf1-8* allele is represented as QE27. The amino acid abbreviations used are as follows: A, alanine; E, glutamic acid; G, glycine; I, isoleucine; L, leucine; Q, glutamine; S, serine.

[b] The generation times and the length of the cell cycle periods were calculated as described in Materials and Methods and by Smith and Stirling (34).

[c] n, number of experiments.

mating ability to determine if the H3 N-terminal domain was also required for inactivation of the silent loci. The results of quantitative mating assays for the wild-type control and the H3 and H4 deletion mutants are presented in Table 2. As expected, cells containing the H4 deletion allele *hhf1-8* mated with an efficiency that was more than 4 orders of magnitude lower than that of wild-type control cells. In contrast, the two H3 deletion alleles resulted in only a 3- to 10-fold decrease in mating ability in the mutants. These experiments show that the N-terminal domain of histone H3, at least through amino acid Ser-28, plays a much less prominent part in silencing transcription from *HML* and *HMR*. Therefore, the roles of the N-terminal domains of histone H3 and H4 are distinct with respect to mating type gene regulation.

Temperature sensitivity. The plating efficiencies of the three mutant strains and the isogenic wild-type control at 23 and 37°C are summarized in Table 3. Cells containing the wild-type genes on the plasmid vector grew normally at both temperatures. Strains expressing either of the H3 N-terminal deletion mutations had high plating efficiencies at 23°C, and both mutants were able to grow at 37°C although with decreased plating efficiency, especially for the larger deletion of H3. In contrast, the strain expressing the deletion derivative of histone H4 was very temperature sensitive for growth and failed to give colonies at 37°C. The plating efficiency of this strain dropped from 46% at 23°C to less than 0.002% at 37°C. The results of several experiments showed that this phenotype was linked to the presence of the *hhf1-8* allele. First, all the colonies isolated from a plasmid shuffle assay using a plasmid containing *hhf1-8* (pMS338) were temperature sensitive. Second, when these cells were transformed with a *URA3/CEN4* plasmid carrying the wild-type histone H3 and H4 genes, the resulting transformants were no longer temperature sensitive for growth at 37°C. Thus, the temperature sensitivity cannot be caused by an unlinked second-site mutation arising during the transformation or plasmid shuffle protocols. Finally, an independent plasmid containing the *hhf1-8* allele in a different vector also gave rise to temperature-sensitive cells.

The growth of the H4 N-terminal deletion mutant during a temperature shift experiment is shown in Fig. 3. While the rate of cell division for wild-type cells increased after the shift to 37°C, the rate of growth of the mutant strain decreased and the culture arrested before one complete cell doubling. There was no change in the cell cycle distribution of the culture arrested at the nonpermissive temperature as judged by cellular and nuclear morphology. Thus, *hhf1-8* does not behave like a classical temperature-sensitive cell division cycle mutation. Transcriptional repression of histone H2B or H4 results in the first-cycle arrest of cells at nuclear division (12, 17; this work). Since *hhf1-8* cells did not show this cell cycle arrest phenotype, their failure to grow cannot be due solely to degradation or loss of histone H4. The survival of the wild-type and mutant cells at 37°C with time are illustrated in Fig. 4. Wild-type cells tolerated the nonpermissive temperature as expected; however, cells expressing the *hhf1-8* allele lost viability at 37°C with a half-life of approximately 2 h.

Double mutants. We next asked whether the double mutant lacking the N-terminal domains of both histones H3 and H4 was alive. To address this question, the histone H4 N-terminal deletion allele was combined with each of the histone H3 deletion alleles generating two plasmids, pMS359 and pMS360 (Materials and Methods). Both plasmids were tested for their ability to support the growth of yeast colonies derived from MX4-22A by plasmid shuffle. Unlike the previous constructs, the double mutant plasmids failed to replace the resident plasmid efficiently. The plating efficiency of cells on 5-FOA medium was approximately 10^{-6}, several orders of magnitude lower than that of wild-type or single-

TABLE 2. Mating efficiencies

Histone allele	Mutation[a]	Relative mating efficiencies[b]	
		*MAT***a**	*MAT*α
HHT1 HHF1	WT	1.0	1.0
hht1-1	Δ(A1–L20)	0.25	0.12
hht1-2	Δ(A1–S28)	0.3	0.1
hhf1-8	Δ(G2–I26), QE27	1.5×10^{-5}	9.2×10^{-5}

[a] The allele designations are described in footnote *a* to Table 1.

[b] The mating efficiencies of the *MAT***a** or *MAT*α cells containing the different N-terminal deletions of H3 or H4. The efficiencies are normalized to that of the wild type, which is set at 1.0.

TABLE 3. Plating efficiencies at different temperatures

Histone allele	Mutation[a]	Plating efficiency[b]	
		23°C	37°C
HHT1 HHF1	WT	109	95
hht1-1	Δ(A1–L20)	87	49
hht1-2	Δ(A1–S28)	101	12
hhf1-8	Δ(G2–I26), QE27	47	<0.002

[a] The allele designations are described in footnote *a* to Table 1.

[b] Cultures were sonicated to produce a single-cell suspension and counted, and then known numbers of cells were plated on YPD medium at 23 and 37°C. Plating efficiencies were calculated as the percentage of single cells that could form colonies on YPD medium at 23 and 37°C.

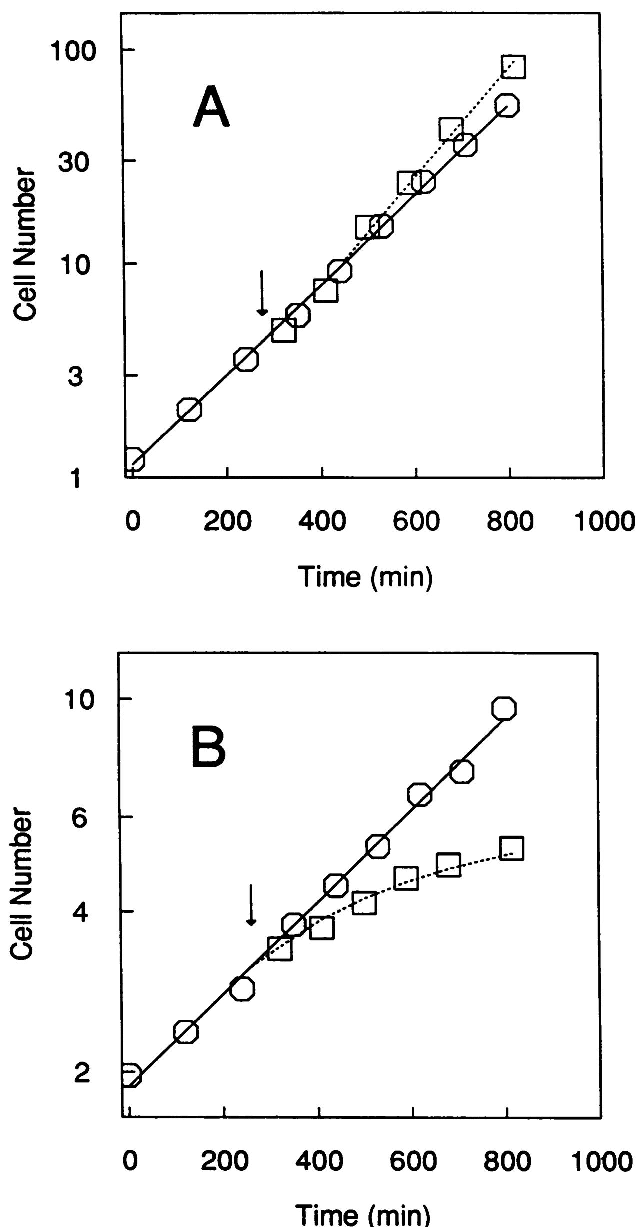


FIG. 3. Growth curves of wild-type and H4 deletion mutant cells. Cell counts were obtained from cultures of strain MX4-22A rescued by pMS337 (*HHT1 HHF1* [A]) or pMS338 (*HHT1 hhf1-8* [B]) growing in YPD medium at 23°C (solid line). At the time indicated by the arrow, half of each culture was shifted to 37°C and cell counts followed with time (dashed line).

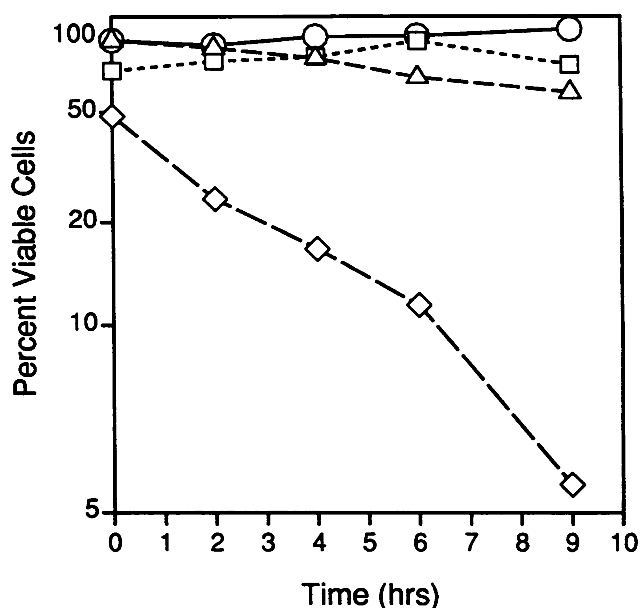


FIG. 4. Survival curves of wild-type and histone deletion mutants. Exponential cultures of MX4-22A in YPD medium rescued by pMS337 (*HHT1 HHF1*) (circles), pMS357 (*hht1-1 HHF1*) (squares), pMS358 (*hht1-2 HHF1*) (triangles), or pMS338 (*HHT1 hhf1-8*) (diamonds) were shifted from 24 to 37°C. At timed intervals after the shift to 37°C cell counts were obtained, diluted to appropriate numbers, and plated on YPD medium at 23°C. Survival is presented as the percentage of cells that could form colonies at 23°C.

mutant plasmids. All of the colonies that grew on 5-FOA medium were picked in each of the double-mutant experiments and examined further by Southern blot analyses. Diagnostic restriction enzyme digests showed that in each case one or both of the mutant alleles had recombined with the wild-type genes on the resident pMS329 plasmid. Therefore, it appeared likely that the simultaneous deletion of the N-terminal domains of histones H3 and H4 was lethal.

The lethality of the double mutant was confirmed with a conditional wild-type histone H4 gene. The plasmid pMS362 carries a fusion of the *GAL1* promoter to the coding DNA of the histone H4 gene *HHF1*. By using this construct, wild-type histone H4 is expressed in cells grown on galactose but is repressed in cells grown on glucose. This fusion gene plasmid was introduced into MX1-4C cells, and those isolates were subsequently transformed with pMS360, a *LEU2* plasmid with the double N-terminal deletion alleles *hht1-2* and *hhf1-8*. Cells were then plated on galactose 5-FOA medium to select for cells that had lost the resident wild-type histone plasmid pMS329, retaining the double N-terminal deletion plasmid. In this case colonies were readily obtained on galactose 5-FOA plates, showing that the wild-type H4 expressed from the galactose promoter could suppress the lethality of the double N-terminal deletion alleles. As expected, these cells died when they were transferred from galactose to glucose medium. These results prove that the simultaneous deletion of the N-terminal domains of both histone H3 and H4 is lethal.

Functions of the N-terminal domains. Two general models for the synthetic lethality of the double N-terminal deletion mutant were considered. In the first model, the N-terminal domains of H3 and H4 were proposed to be required for nucleosome formation; in the absence of both the H3 and H4 N-terminal domains the cells would become depleted for nucleosomes. In the second model, nucleosome formation was proposed to be normal, but the resulting chromatin was then defective for one or more essential functions. These two models could be distinguished because the first predicted that the double deletion mutant would have a nucleosome depletion phenotype similar to that known to occur for histone H2B or H4 null mutants (12, 17).

Two strains derived by plasmid shuffle were compared in the conditional H4 expression system. Each strain contained the *GAL1::HHF1* fusion plasmid pMS362 to provide wild-type histone H4 when the cells were grown on galactose.

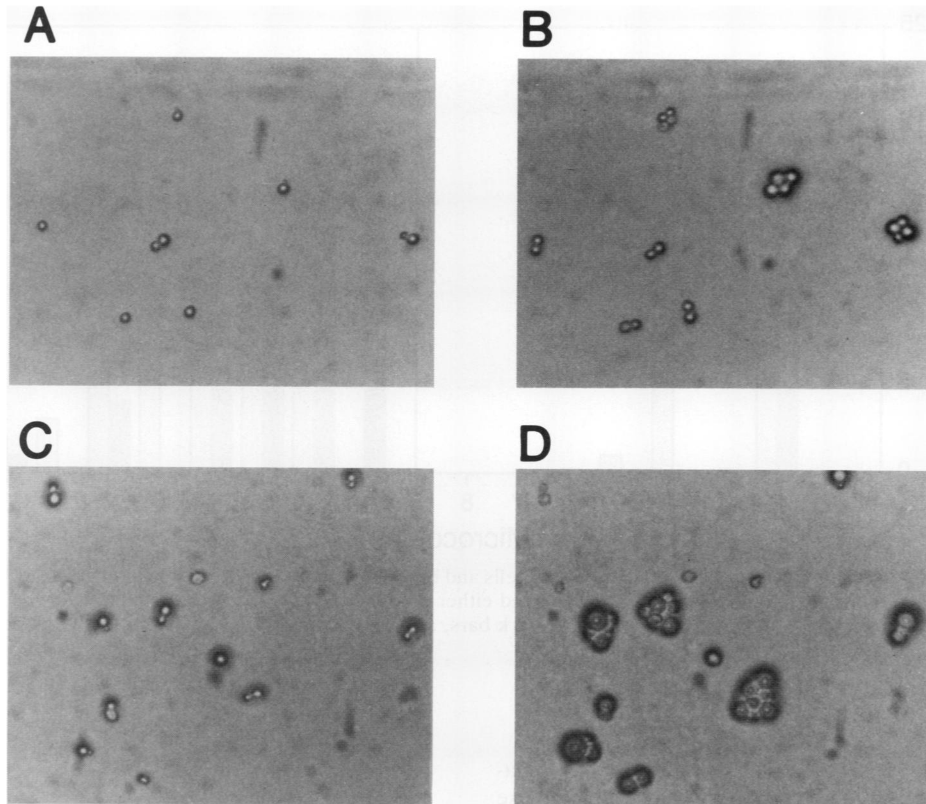


FIG. 5. Microcolony assays of histone mutants. Cells from cultures of strain MX1-4C containing pMS362 (*GAL1*::*HHF1*) and either pMS347 (*HHT1*) (A and B) or pMS360 (*hht1-2 hhf1-8*) (C and D), were grown in galactose (YPG) medium at 28°C, sonicated, and then plated on glucose (YPD) medium at 28°C. Representative regions of the YPD plates were photographed at the time of plating (A and C) and after 24 to 36 h (B and D).

The first strain also contained a *LEU2* plasmid pMS347 that contained only the wild-type histone H3 gene. This strain was able to grow on galactose medium but was unable to grow on glucose medium because histone H4 expression was completely repressed. The second strain was described in the previous section and contained the *LEU2* plasmid pMS360 with the N-terminal deletion alleles *hht1-2* and *hhf1-8*.

The growth phenotype of each strain was examined following repression of wild-type histone H4 transcription on glucose. Asynchronous cultures were grown to early exponential phase in galactose medium at 28°C and then shifted to glucose medium. The results obtained with the H4 null mutant strain were in complete agreement with those of Kim et al. (17) for histone H4 depletion. Cultures containing pMS347 arrested growth after 1.6 to 1.7 cell divisions, at which point more than 95% of the cells had a 2C DNA content. In contrast, the double deletion mutant containing pMS360 arrested growth after 2.3 to 2.4 cell divisions following the shift to glucose medium. Flow cytometry results and the cellular and nuclear morphologies of cells with the double N-terminal deletion mutations showed that they arrested throughout the cell division cycle.

Microcolony assays (40) were performed to determine the fates of individual cells. For these assays, cells containing the *GAL1*::*HHF1* fusion plasmid, pMS362, and either pMS347 or pMS360 were grown to early log phase in galactose medium at 28°C, sonicated to produce a single-cell suspension, and then plated on glucose medium at 28°C. Fields of cells were photographed at the time of plating and again 24 to 36 h later (Fig. 5). The histograms of "cell plus bud" counts for the two strains are compared in Fig. 6. In the case of cells containing pMS347, the H4 null mutant, unbudded cells arrested in the first division cycle with a single large bud. Budded cells generally completed their current division cycle and arrested in the next cycle as two cells, each with a single large bud (Fig. 5A and B and 6).

The phenotype of the double N-terminal deletion mutant was quite different. When shifted to glucose medium, cells carrying pMS360 were able to undergo several rounds of cell division before arresting (Fig. 5C and D and 6). The distribution of microcolony sizes was consistent with first-order decay kinetics, suggesting a stochastic component to the cell death (Fig. 6). The terminal morphology of the cells on glucose was heterogeneous and did not show a cell division cycle phenotype. Approximately 27% of the microcolonies that completed multiple divisions contained one or more cells or buds with a fragile cell wall. These fragile cells were detectable in phase microscopy and were easily disrupted by micromanipulation with a glass needle.

Some of the microcolonies formed from single unbudded cells of the double deletion mutant had colony sizes of three or six cells (Fig. 6), suggesting that the mother and daughter lineages in a colony did not always arrest division in the same generation. To determine if cell division was restricted to either the mother or daughter cell lineage, pedigrees were determined for progeny of single cells. Unbudded cells were transferred from galactose medium to a glucose agar slab,

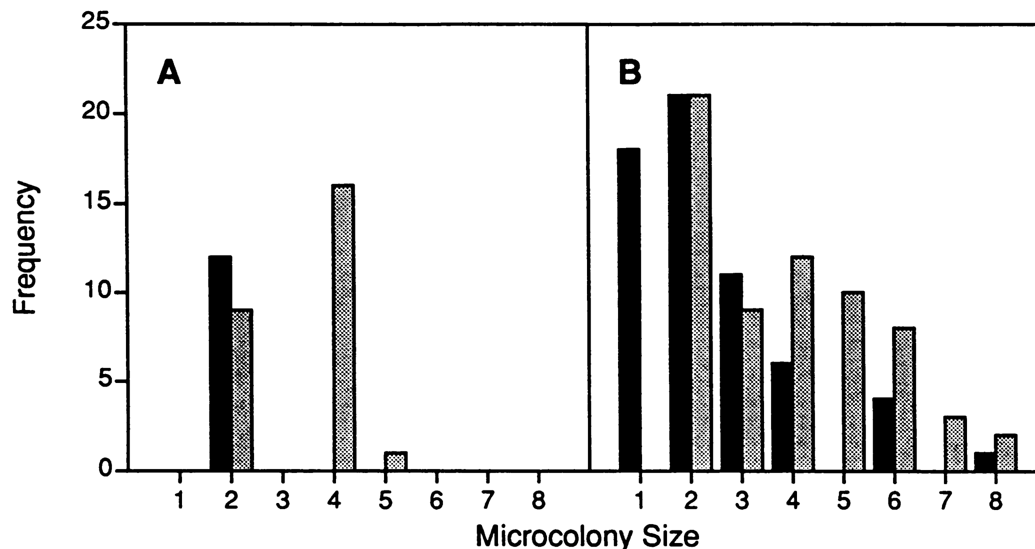


FIG. 6. Histogram of microcolony sizes. The total number of cells and buds were scored after 24 to 36 h on glucose (YPD) medium at 28°C for cells of strain MX1-4C containing pMS362 (*GAL1::HHF1*) and either pMS347 (*HHT1*) (A) or pMS360 (*hht1-2 hhf1-8*) (B). The sizes of colonies from cells that were initially unbudded are shown by dark bars, and those from cells that were initially budded are shown by light bars.

and mother and daughter cells were separated by micromanipulation during growth and division. The results of these experiments showed that both mother and daughter cell lineages were capable of a limited number of successive cell divisions. For cells that arrested in the second division cycle, approximately 55% of the daughter and 27% of the mother cells initiated a new bud. Two pedigrees that proceeded partially into the third division cycle are shown in Fig. 7.

In summary, expression of the H3 and H4 N-terminal deletions in the double mutant caused cells to arrest with a phenotype that was very different from that produced by histone H4 depletion. These results show that the synthetic lethality of the double mutant is not solely due to a lack of histone. They suggest instead that the chromatin assembled from H3-H4 tetramers lacking their N-terminal domains is functionally defective for one or more essential functions.

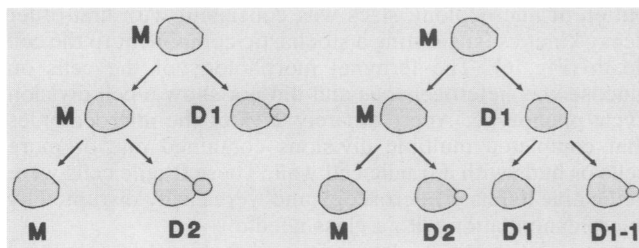


FIG. 7. Pedigree analysis. The pedigrees derived from two cells containing the double N-terminal domain deletion alleles of H3 and H4 are shown. Single unbudded cells of strain MX1-4C containing pMS362 (*GAL1::HHF1*) and pMS360 (*hht1-2 hhf1-8*) were transferred from galactose (YPG) medium to a glucose (YPD) agar slab and followed by micromanipulation. The fates of mother cell lineages (M) are drawn to the left, and those of daughter cell lineages (D) are drawn to the right.

DISCUSSION

The results of the experiments reported here support several conclusions: (i) individually neither the H3 nor the H4 N-terminal domain is required for cell viability in yeast, (ii) the H3 and H4 deletion mutants share some but not all phenotypes, (iii) the simultaneous deletion of the N-terminal domains of both H3 and H4 results in a synthetic lethality, and (iv) the N-terminal domains of H3 and H4 complement each other for one or more essential function(s) required throughout the division cycle. Combining the results reported here with previous results for histones H2A and H2B (28, 39) and H4 (16, 20), we can conclude that the N-terminal domains of all four core histone proteins are individually dispensable for cell viability.

All three N-terminal deletion mutants described here had increases in the length of the S and G_2+M phases of the cell division cycle. Similar increases were seen in the length of S and G_2+M phases of an H4 allele, *hhf1-10*, in which the four lysine residues subject to reversible acetylation were replaced with glutamine residues, simulating the hyperacetylated state (20). Histone H3 also has five lysines in its N-terminal region that are sites of reversible acetylation (19). The G_2+M defect shared by all these mutants is characteristic of two general classes of mutants, those involving DNA damage and those affecting chromosome segregation (40). At present we cannot distinguish between these two possibilities; however, several observations suggest that the major defect may be in segregation. Deletion of the *RAD9* gene does not alter the first-cycle G_2 arrest of cells in which histone H4 expression is repressed, indicating that the block is not caused solely by DNA damage (unpublished data). Conversely, arginine-to-lysine point mutations in the N-terminal domain of H4 increased the length of S phase, equivalent to that observed for the three N-terminal deletion mutants, but failed to increase the length of the G_2+M periods (20). Finally, flow cytometry histograms of DNA content for the N-terminal deletion mutants show an accu-

mulation of aneuploid cells, suggesting defects in chromosome segregation.

The histone H4 N-terminal deletion mutant has at least two phenotypes that are not shared with the H3 deletion mutants: mating sterility and temperature-sensitive lethality. This temperature sensitivity can be separated from the mating sterility by appropriate point mutations (20, 20a). The silencer function of the N-terminal domain of H4 maps to a region spanning amino acids 16 to 18 (15, 20, 25). This sequence of histone H4 has homology with residues 23 to 27 of histone H3 in both primary sequence and secondary modifications (Fig. 1A) (5). Because of this similarity we anticipated that *hht1-2* but not *hht1-1* would show mating sterility; however, neither deletion had a major effect on mating type expression in either *MAT*a or *MAT*α strains.

The microcolony assays described here revealed that cells burdened with the simultaneous deletion of the N termini of histones H3 and H4 were capable of a limited and variable number of cell divisions, ultimately arresting at random with variable morphologies. We propose that cells of the double deletion mutant are viable on galactose medium, when wild-type H4 expression is induced, because an essential function(s) of the chromosome is assembled into nucleosomes containing one or more histone H4 N-terminal domains. However, once the cells are transferred to glucose and wild-type histone H4 expression is repressed, only deletion tetramers of H3 and H4 can be assembled. The pattern of random death of the double deletion cells reflects the random assembly of defective H3-H4 tetramers onto an essential region(s) of the chromosomes. The variety of phenotypes and morphologies observed in arrested cells is consistent with the disruption of different sets of essential functions in each.

The microcolony and pedigree results for the double deletion mutant are incompatible with complete conservative inheritance of nucleosomes during chromosome replication. If one sister chromatid retained only old nucleosomes and the other was assembled with only newly synthesized nucleosomes, then cells shifted to glucose would always have one chromatid assembled into defective chromatin with H3-H4 deletion tetramers. Thus, even if there were only a single essential function sensitive to the assembly of H3-H4 deletion tetramers, at least one of the progeny cells of each division would always be programmed to die. However, the pedigree results of cells shifted to glucose are incompatible with this prediction (Fig. 7). Thus, these results provide the first genetic evidence supporting the random inheritance of nucleosomes during chromosome replication, at least over the essential function(s) assayed by viability. For a recent review of biochemical experiments on nucleosome inheritance, see Svaren and Chalkley (35).

The deletion of amino acids 1 to 20 of H3 (*hht1-1*) precisely mimics the previously reported in vivo cleavage of the N terminus of H3 by the FMDV 3C protease (11). Expression of a cDNA clone encoding the FMDV 3C protease has been reported to inhibit host cell transcription, suggesting that removal of the first 20 amino acids of H3 may be responsible for the block (36). However, deletion of amino acids 1 to 20 of H3 is not lethal in *S. cerevisiae*, and if there is an inhibition of RNA synthesis it cannot be a complete block, since the cells continue to grow. Alternatively, perhaps the N-terminal peptide released by 3C proteolytic cleavage could inhibit RNA synthesis directly.

ACKNOWLEDGMENTS

We thank our colleagues for help and discussions, particularly Pablo Grigera for providing data on the proteolysis of histone H3 by the FMDV 3C protease prior to publication. We also thank Chris Eichman for expert technical assistance with the flow cytometry.

This work was supported by National Institutes of Health grant GM28920, awarded to M.M.S.

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