Histone H3 and H4 Gene Deletions in *Saccharomyces cerevisiae*

M. Mitchell Smith and Vivianne B. Stirling

Department of Microbiology, School of Medicine, University of Virginia, Charlottesville, Virginia 22908

Abstract. The genome of haploid *Saccharomyces cerevisiae* contains two nonallelic sets of histone H3 and H4 genes. Strains with deletions of each of these loci were constructed by gene replacement techniques. Mutants containing deletions of either gene set were viable, however meiotic segregants lacking both histone H3 and H4 gene loci were inviable. In haploid cells no phenotypic expression of the histone gene deletions was observed; deletion mutants had wild-type growth rates, were not temperature sensitive for growth, and mated normally. However, diploids homozygous for the H3-H4 gene deletions were slightly defective in their growth and cell cycle progression. The generation times of the diploid mutants were longer than wild-type cells, the size distributions of cells from exponentially growing cultures were skewed towards larger cell volumes, and the G1 period of the mutant cells was longer than that of the wild-type diploid. The homozygous deletion of the copy-II set of H3-H4 genes in diploids also increased the frequency of mitotic chromosome loss as measured using a circular plasmid minichromosome assay.

I N all of the eukaryotes studied to date the genes encoding the histones are organized as multigene families. The arrangement of these gene families may be quite complex, as in metazoan cells, or relatively simple, as in the single-cell eukaryotes (20, 29). Both highly ordered tandem arrays of histone gene repeats and randomly dispersed genes, gene pairs, or clusters exist, and sometimes coexist, in various species. Histone gene copy number can vary widely among species without any apparent phylogenetic pattern. The roles of these multiple histone genes in cellular function and development are not well understood.

The tandem repeat histone gene families provide dramatic examples of increased copy number. Most of the several hundred sea urchin histone genes belong to the major tandem repeat family expressed during cleavage-stage embryos (20). However, the precise number of active genes within the tandem repeat clusters is not known and their role in the development of the sea urchin remains enigmatic (28, 30, 32). The tandem repeat families aside, even the number of disperse repeated genes for a particular histone can approach 10–20 copies per haploid genome. In many cases these multiple genes are known to be expressed in the cell since sequence divergence in the transcribed noncoding regions of the genes can be detected in the RNA population (22, 25, 32). Furthermore, DNA sequence comparisons of some of the disperse duplicated genes indicate that multiple functional genes have been conserved during evolution over long periods of time — perhaps as long as 500 million years (29).

In certain cases, the histone family members may fall into distinct primary sequence variants that demonstrate particular profiles of expression throughout the course of embryonic

development or even within the period of the cell division cycle (2, 5, 6, 8, 16, 25, 32, 51). While these correlations suggest that histone variants perform specific functions in development or cell growth, there is as yet no direct experimental evidence that these variants are required for normal function or play distinct roles in the cell.

The histone genes of *Saccharomyces cerevisiae* are arranged as dispersed duplicate copies of specific gene pairs (45). In the haploid genome there are two nonallelic loci composed of paired H2A and H2B genes, and two additional nonallelic loci composed of paired H3 and H4 genes (21, 47). The four genetic loci are unlinked and all eight genes are transcribed and encode functional histones. The duplicated H3 and H4 genes encode identical histones H3 and H4, while the duplicated H2A and H2B genes encode protein variants (7, 46, 49). This relatively simple gene arrangement, and the well-developed techniques available for molecular genetics in *S. cerevisiae*, make possible an experimental test of the role of gene copy number and protein subtypes in vivo. Experiments on the H2A and H2B genes of yeast have demonstrated that the duplicate genes and their protein variants are functionally redundant (27, 34, 39). Cell viability is dependent upon at least one copy of the H2A and H2B genes but either subtype gene is sufficient for most, if not all, mitotic and meiotic functions. Furthermore, the H2A and H2B subtype genes can be freely interchanged between sets in any combination.

We have investigated the role of the duplicate H3-H4 loci in the cell by gene deletion experiments. The genetic experiments reported here demonstrate that, like the H2A and H2B genes, the individual H3-H4 loci are dispensable for cell viability. As expected, at least one set of genes is essential. Finally, we have investigated the effects of the H3-H4 gene

Table I. Yeast Strains

Strain	Relevant markers	Source
YP3	a *ade2-101 lys2-801 ura3-52* α *ade2-101 lys2-801 ura3-52*	P. Hieter
MSY159	a *ade2-101 lys2-801 ura3-52*	This work
MSY155	a *ade2-101 lys2-801 ura3-52* Δ*(HHT1 HHF1)*	This work
MSY156	α *ade2-101 lys2-801 ura3-52* Δ*(HHT1 HHF1)*	This work
MSY157	a *ade2-101 lys2-801 ura3-52* Δ*(HHT2 HHF2)*	This work
MSY158	α *ade2-101 lys2-801 ura3-52* Δ*(HHT2 HHF2)*	This work
MSY164	a *ade2-101 lys2-801 ura3-52* Δ*(HHT1 HHF1)* α *ade2-101 lys2-801 ura3-52* Δ*(HHT1 HHF1)*	This work
MSY165	a *ade2-101 lys2-801 ura3-52* Δ*(HHT2 HHF2)* α *ade2-101 lys2-801 ura3-52* Δ*(HHT2 HHF2)*	This work
MSY167	a *ade2-101 lys2-801 ura3-52* α *ade2-101 lys2-801 ura3-52*	This work

deletions on the growth, cell division cycle, mitotic chromosome transmission, and life cycle of mutant cells.

Materials and Methods

Strains and Plasmids

The *Escherichia coli* host strain for all bacterial transformations was ED8654 (*supE supF hsdR⁻ hsdM⁺ met⁻ trpR*) (33). The yeast strains used are listed in Table I along with relevant genetic markers. For genetic designations the copy-I histone H3 and H4 genes are called *HHT1* and *HHF1*, respectively, and the copy-II genes are called *HHT2* and *HHF2*. Standard culture conditions and techniques were used for growth and manipulation of the yeast (40). The H3-H4 histone gene plasmids pMS191, pMS201, and pMS202 have been described previously (47). The yeast-*E. coli* shuttle vector YRp14 is a chimeric plasmid composed of pBR322, the yeast selectable marker gene *URA3*, and the yeast ochre supresor gene *SUP11* (48). The fragment Sc207, carrying the Eco RI fragment flanking the copy-II H4 gene, was isolated by its ability to hybridize with pMS202. It was cloned from a yeast genomic library of D649 constructed in the bacteriophage lambda vector λNEM641 (33). The mitotic segregation plasmid, pAB9SEG, used in the color colony assays for chromosome stability has been described previously (3). It is a derivative of YRp14CEN4 that contains a 374-base pair (bp) Sau3AI fragment carrying the efficient autonomously replicating segment (ARS)[1] located downstream of the copy-I histone H4 gene (3, 4).

In Vitro Deletions

The organization of the H3-H4 gene sets and their deletion derivatives is summarized in Fig. 1. The copy-I deletion was constructed by Bal 31 nuclease digestion at the unique Sma I site in the intergene region between the H3 and H4 genes. A set of Bal 31 digestion fragments was selected by screening for fragment size on agarose gels and the DNA sequences of the breakpoints were determined from M13 phage subclones of the fragments. The leftward deletion breakpoint ends 186 bp downstream of the end of the H3 transcript and 21 bp before the end of the open reading frame of the *SMT1* gene. The rightward deletion breakpoint ends 185 bp past the end of the H4-transcribed sequences and 73 bp past the histone ARS core consensus sequence (3, 4). These left and right deletion fragments were joined by an Eco RI synthetic DNA linker in the recombinant.

The copy-II deletion was constructed from restriction fragments flanking the H3 and H4 genes. The lefthand section was a Hind III-Acc I fragment from the yeast histone H3 clone Sc201 while the righthand section was the Hind III-Eco RI fragment Sc218 that is adjacent to the H4-distal righthand end of the yeast histone H4 clone Sc202 (Fig. 1). These two restriction fragments were joined by a synthetic Bam HI linker.

In Vivo Deletions

The histone H3-H4 deletion constructs were introduced into the chromo-

1. *Abbreviation used in this paper*: ARS, autonomously replicating segment.

some using the shuttle vector YRp14 and a protocol as previously suggested (23, 48). The YRp14 vector carried the *SUP11* ochre suppressor and thus the integrated plasmid could be detected by suppression of *ade2-101* in the host yeast. This permitted the construction of a diploid that was homozygous for the integration of the shuttle plasmids at the histone loci. Diploid strain YP3 was transformed to *URA⁺* with YRp14 plasmid derivatives containing the copy-I or copy-II deletion constructs. The transformants were pink as a result of partial suppression of the homozygous diploid *ade2-101* alleles by one copy of the *SUP11* plasmid gene. Isolates were sporulated and tetrads were dissected to yield two red and two white spore colonies. White segregants were the result of complete suppression of the single haploid *ade2-101* allele by the plasmid *SUP11* gene. The white colonies were screened for isolates that carried the YRp14-histone deletion plasmids integrated at the histone loci by Southern blot hybridization analysis. From this set, a and
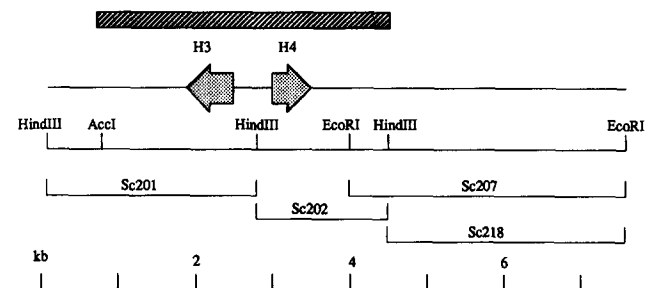
Copy-I
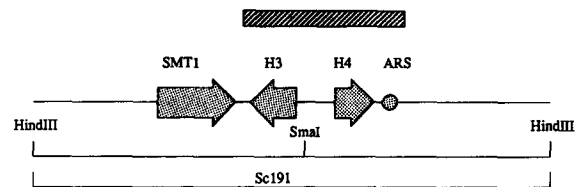
Copy-II

Figure 1. Organization of the histone H3-H4 gene loci. The locations of the histone H3 and H4 genes are shown for the two nonallelic loci with respect to cloned DNA fragments. The positions of an unrelated gene *SMT1*, and the autonomous replication element, ARS, are also shown for the copy-I locus. The extents of H3-H4 gene deletions are indicated by the hashed bars over each of the maps. Each deletion removes the entire transcribed region for the H3 and H4 genes. For the copy-I locus, the *SMT1* gene was left intact but the ARS element was removed.

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α haploids were mated to give white diploids homozygous for the integrated shuttle plasmid.

The white homozygous diploids were grown on YPD plates containing 1.5 M ethylene glycol to select for those cells in which the integrated shuttle vector had excised (13, 42). These cells gave pink colonies because of the loss of one copy of *SUPI1*. Pink diploids were again sporulated and tetrads dissected to give two red and two white spore colonies. The red colonies identified meiotic segregants that inherited the chromosome in which the shuttle plasmid had excised. Genomic DNA from these red isolates were screen by Southern blot analysis to detect the cells in which the excision of the shuttle plasmid left the histone gene deletion in the chromosome.

Cell Growth and Flow Microfluorometry

For growth measurements, cells were removed from cultures at timed intervals and the samples were sonicated for 20 s to obtain single cells. Cell counts were obtained using a ZM particle counter (Coulter Electronics, Inc., Hialeah, FL). Generation times were estimated by a nonlinear least squares fit to the exponential portion of the growth curve (24). Cell volume distributions were collected using a C256 Channelizer pulse height analyzer (Coulter Electronics, Inc.) connected to the ZM particle counter.

Cells for flow cytometry were fixed and stained with mithramycin as described by Slater et al. (44). Histograms of relative fluorescence for DNA content were collected using an EPICS fluorescence activated cell sorter (Coulter Electronics, Inc.). The mithramycin was excited with the laser set at 457-nm and 250-mW output. The histograms of DNA distribution were analyzed using a nonlinear least squares fit to multiple Gaussian populations (14). The data were modeled as four functions, one for each of the G1, S, and G2 periods and a fourth for the small fraction of aggregated cells remaining in the sample. The four Gaussian functions were constrained to have a constant ratio of mean to standard deviation. The resulting 10-parameter model was fit using a nonlinear curve fitting program (24).

Mitotic Chromosome Stability

Mitotic chromosome transmission was measured using the color colony assay of Hieter et al. (23). The wild-type diploid, MSY167, and the two histone deletion diploids, MSY164 and MSY165, were each transformed with the minichromosome plasmid pAB9SEG. Cells were grown for 4 h in YPD medium and then spread on complete synthetic medium containing a limiting amount of adenine at 2 mg/liter. The plates were incubated at 28°C and then scored for colony color phenotype and sectoring.

Results

Deletion of the H3–H4 Genes

Histone H3 and H4 gene deletion plasmids were constructed in vitro as described in Materials and Methods. The structures of the resulting H3-H4 gene deletion fragments are summarized in Fig. 1. The copy-I locus is complex and in addition to the histone genes it carries a closely linked gene designated *SMT1* (47). At present it is not known if *SMT1* is an essential gene and therefore care was taken not to disrupt it in the deletion construction. The copy-I locus also carries an autonomously replicating segment, or ARS, 3' of the histone H4 gene (3, 4). ARS elements are putative origins of DNA replication (12, 50) and have also been found linked to the yeast histone H2A-H2B gene loci (35, 36). The rightward deletion breakpoint was chosen to remove the ARS so that it could be replaced in the genome with experimental ARS mutations in future experiments. The final copy-I deletion construction therefore resulted in the excision of 2081 bp of chromosomal DNA and extended from nucleotides 2,587 to 4,669 on the coordinates of the Scl91 fragment.

The copy-II locus is not as complex. Aside from the H3 and H4 genes, no other transcripts have been detected in the region (47) and functional studies have ruled out the presence of an ARS near the copy-II histone genes (4). Therefore, the copy-II locus deletion was constructed from suitable restriction fragments cloned from the region. The final copy-II deletion removes ~3.7 kb of chromosomal DNA (Fig. 1).

Transplacement of Deletions into Yeast

The deletion plasmids made in vitro were next used to replace the chromosomal copy-I and copy-II loci in vivo. The plasmids were transformed into the diploid strain YP3 so that the deletion would be complemented if copies of both sets of H3-H4 loci were essential for viability. The histone deletions were shuttled into the genome as described in Materials and Methods.

Tetrads from diploid strains in which the deletion shuttle plasmid had excised from one chromosome were dissected. These dissections gave tetrads with four viable spores producing two white and two red spore colonies. The red colonies identified haploids in which the plasmid, and thus the *SUPI1* gene, had recombined out of the chromosome. This excision results in one of two outcomes: either the wild-type sequences are retained at the locus, or the deletion construction replaces the wild-type genes. Genomic DNA preparations from several red colonies were screened for the histone gene deletions by Southern blot analysis. In this way deletions of each of the H3-H4 loci were identified and confirmed by restriction endonuclease mapping.

Sets of diagnostic Southern blots are presented in Fig. 2 and demonstrate that the in vitro deletions were correctly shuttled into the corresponding histone loci. In the copy-I results of Fig. 2 *A*, for example, the Hind III digestions of whole genome DNA from wild-type and copy-II deletion strains show the normal 6.8-kb copy-I restricton fragment when probed with Scl91. However, in the copy-I deletion strain this fragment is ~2 kb smaller. The other restriction enzyme digestions in Fig. 2 *A* show that the deletion spans the H3- and H4-transcribed sequences and that a new Eco RI linker has been inserted at the site of the deletion. Since the Scl91 fragment used as the probe in Fig. 2 *A* contains the H3 and H4 coding sequences, it also crosshybridizes with the coding sequences of the copy-II locus. These copy-II fragments are present in the DNA digests from wild-type and copy-I deletion strains, but are absent in the copy-II deletion strain indicating that the coding sequences are missing from the putative copy-II deletion. Positive evidence for the deletion is presented in Fig. 2 *B*. The hybridization probe in this case was the Sc218 fragment that is specific for the copy-II downstream sequences. In the DNA from the copy-II deletion strain the normal 3.5-kb Sc207 Eco RI fragment is fused with an H3-distal fragment by the deletion to a give a 9.0-kb fragment seen in the Eco RI digestions, while it is truncated with the new Bam HI linker at the site of the deletion to give a 3.0-kb fragment seen in the Eco RI–Bam HI double digestions.

Thus, haploids containing deletions of either the copy-I or the copy-II histone H3-H4 gene pairs were successfully constructed. If deletion of either the copy-I or copy-II genes had been lethal, dissection of the pink variant diploids would have produced two live white spore colonies and two dead spores from the segregants with the deletion chromosome. We conclude that the copy-I and copy-II histone H3-H4 loci are functionally redundant; the presence of either region alone is sufficient for cell viability. This is in agreement with the results for disruption of the duplicated H2A-H2B loci (27, 39).
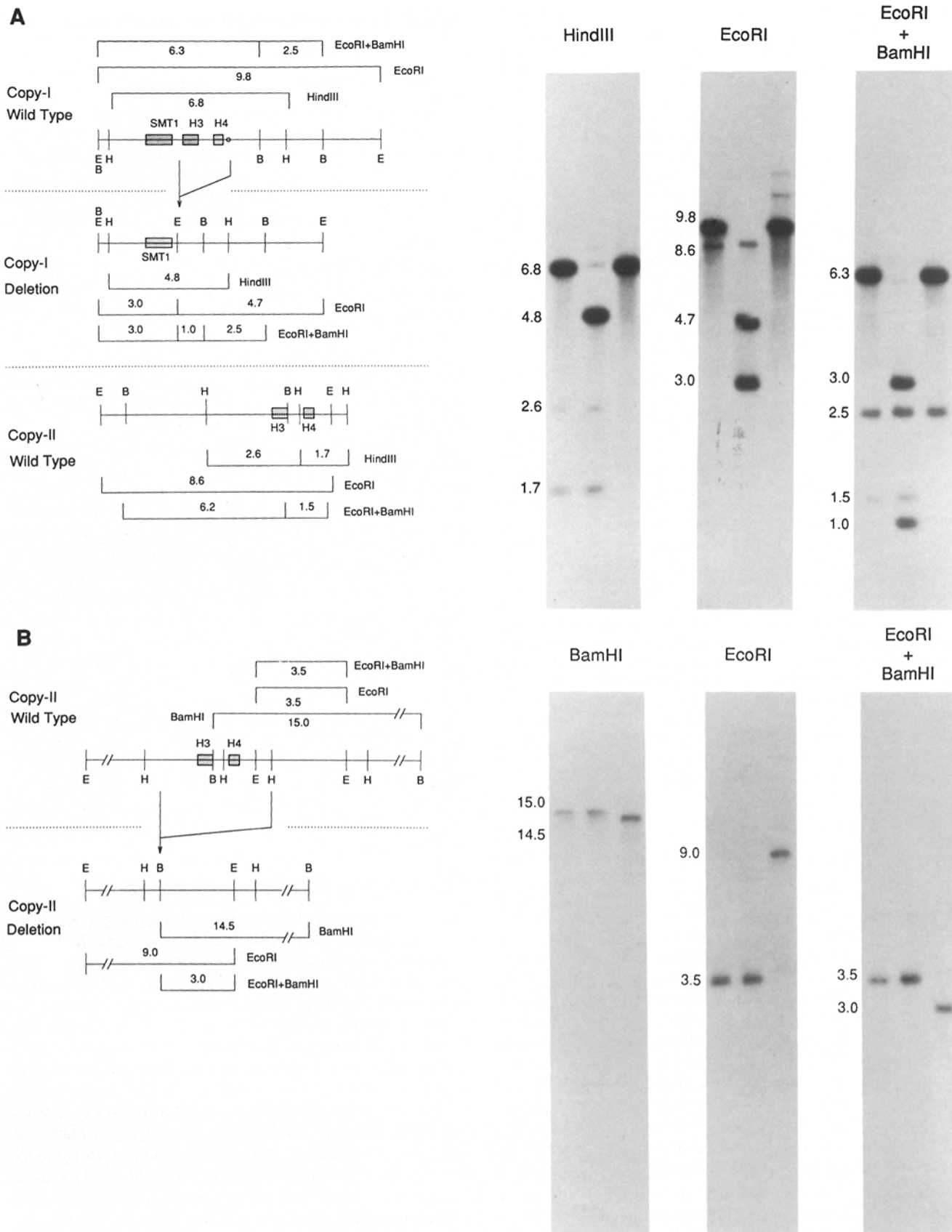
Figure 2. Restriction map analysis of the histone H3-H4 gene loci in yeast deletion mutants. The autoradiographs to the right of each panel show the pattern of hybridization to restriction enzyme digests of total DNA from the three strains MSY167, MSY155, and MSY157. In each case the group of three DNA samples were loaded, left to right, MSY167 (wild type), MSY155 (copy-I deletion), and MSY156 (copy-II deletion). The sizes of the hybridizing restriction fragments, in kilobase pairs, are shown to the left of each autoradiograph. The diagrams
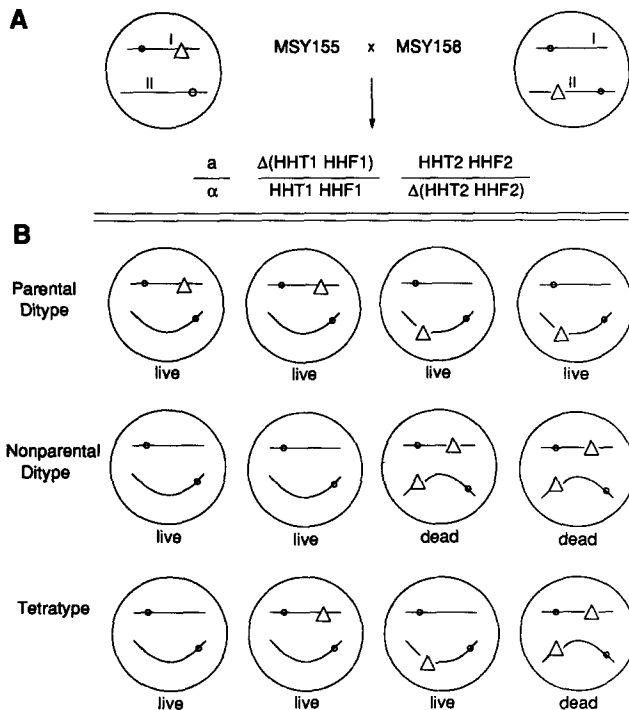
A

MSY155 × MSY158

a	Δ(HHT1 HHF1)	HHT2 HHF2
α	HHT1 HHF1	Δ(HHT2 HHF2)

B

Parental Ditype

live live live live

Nonparental Ditype

live live dead dead

Tetratype

live live live dead

Figure 3. Tetrad analysis of histone deletions. (*A*) The relevant features of a cross between the copy-I deletion strain MSY155 and the copy-II deletion strain MSY158 are shown. Two linkage groups are illustrated in each "cell" representing the unlinked copy-I and copy-II histone H3-H4 loci. The deletions are represented by the open triangles on each linkage group. The cross results in a diploid that is hemizygous for each H3-H4 gene set. (*B*) The segregation results expected for the cross are shown for each class of tetrad. If at least one copy of either set of histone H3 and H4 genes is essential, then the tetrad analysis will show a pattern in which spores inheriting both gene deletions will be dead. That is, tetrads giving two live and two dead spores should be nonparental ditype, those giving three live spores and one dead should be tetratype, and those giving four live spores should be parental ditype.

The H3-H4 Genes Are Essential

The results presented above demonstrate that one histone H3-H4 gene set is sufficient for cell viability. We next tested whether at least one set of genes is essential for growth. A haploid copy-I deletion strain was mated with a haploid copy-II deletion strain to produce a diploid that was hemizygous for each of the H3-H4 gene loci. This diploid was sporulated and ascii were dissected for tetrad analysis. The predicted results for this dissection are diagrammed in Fig. 3. If at least one copy of the H3 and H4 genes is essential for growth, then nonparental ditype tetrads should produce only two viable spores, tetratype tetrads should produce three live spores, and parental ditype tetrads should produce four live spores. The experimental dissection yielded tetrads having the expected range of spore viabilities. From a total of 54 tetrads,

Table II. Tetrad Analysis of Histone Gene Deletions

Viable spores	Tetrad type		
	Parental ditype	Nonparental ditype	Tetratype
n			
2	0	6	0
3	0	0	4
4	2	0	0

the numbers of four, three, and two viable spore tetrads were 16, 18, and 13, respectively. Five tetrads gave only one viable spore each and two tetrads were completely inviable. The approximate 1:1 ratio of parental to nonparental ditypes is as expected if the two H3-H4 loci are unlinked to each other. The low frequency of tetratype tetrads suggests that both loci are linked to their respective centromeres. Experiments to genetically map both loci are in progress.

Genomic DNA was prepared from a selection of two, three, and four viable spore tetrads, and the genotype of the histone H3-H4 loci were scored by Southern blot analysis. Tetrads with two viable spores were preferentially chosen since only two DNA preparations were needed to score an event. The results are presented in Table II. All of the scored tetrads with four viable spores were parental ditype for the copy-I and copy-II loci; that is, two spore colonies had the copy-I wild-type gene set, and two had the copy-II wild-type gene set. All of the three spore tetrads were tetratype for the histone genes, and the missing spore would have carried a deletion of both the copy-I and the copy-II loci. Finally, all of the two spore tetrads scored were nonparental genotypes and each of the two surviving spore colonies carried both of the wild-type H3-H4 loci. In all cases, genetic segregants that would have contained deletions of both sets of histone H3 and H4 genes failed to grow. Therefore, from these experiments we conclude that haploid cells must have at least one of the histone H3-H4 gene sets, but that they need not have both sets.

Growth of Deletion Haploids

We next investigated the growth and cell cycle properties of the viable histone deletion strains. Although the duplicated H3-H4 loci were functionally redundant for cell viability, the deletions need not have been phenotypically neutral. We first looked at the behavior of the haploid deletion cells.

Strains deleted for either the copy-I or copy-II genes had normal morphology and were neither temperature nor cold sensitive for growth. The wild-type, copy-I deletion, and copy-II deletion haploids were grown in YPD and synthetic complete minimal liquid culture, and their growth rates were measured. The doubling times for the strains are shown in Table III. The growth rates in YPD medium were identical for all three strains. Generation times were also very similar

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on the left of each panel illustrate the expected restriction fragments for the relevant genotypes and enzyme digestions. (*A*) The Southern blots were probed with the copy-I Hind III fragment Sc191 (see Fig. 1). Since Sc191 includes the coding DNA of the H3 and H4 genes, it will hybridize with the copy-II H3 and H4 coding sequences as well as the entire copy-I Sc191 region. Thus, the bands in the autoradiographs are derived from either the wild-type copy-I locus, the copy-I deletion locus, or the wild-type copy-II locus. (*B*) The Southern blots were probed with the copy-II-specific fragment Sc218 (Fig. 1). This fragment does not hybridize with sequences at the copy-I locus. Therefore, the bands in the autoradiographs were derived from either the copy-II wild-type locus or the copy-II deletion locus.

Table III. Growth Rates of Wild-type and Histone Deletion Strains

| Strain | Histone H3-H4 genotype | Doubling time | |
		SDC*	YPD‡
		min	min
MSY159	Wild type	90 ± 4	84 ± 4
MSY155	Copy-I deletion	93 ± 3	86 ± 3
MSY157	Copy-II deletion	94 ± 3	85 ± 3
MSY167	Wild type	91 ± 2	84 ± 6
MSY164	Copy-I deletion	103 ± 3	89 ± 2
MSY165	Copy-II deletion	99 ± 3	92 ± 4

* Synthetic dextrose complete medium (40).
‡ Yeast peptone dextrose medium (40).

in synthetic medium and the deletion haploids grew no more than 5% slower than wild-type cells. Both a- and α-haploid strains for either copy-I or copy-II gene deletions were capable of mating normally with haploids of the opposite mating type. In short, no significant macroscopic phenotype was detected for the deletion of either histone H3-H4 gene set.

Sporulation of Deletion Diploids

The haploid histone H3-H4 deletion strains were next mated to construct a set of three diploid yeasts. MSY164 is homozygous for the deletion of the copy-I locus, while MSY165 is homozygous for the deletion of the copy-II locus. The diploid MSY167 is wild type for both of the histone loci. These strains were examined for their ability to sporulate and all three diploids were found to be able to sporulate on acetate medium. No significant differences in the percentage of ascii were observed; the results for each strain were 37.4 ± 5.1 for MSY167, 33.5 ± 4.3 for MSY164, and 39.1 ± 2.3 for MSY165. The time course of sporulation was also the same for the three strains.

Growth of Deletion Diploids

The diploid deletion and wild-type strains were also assayed for mitotic growth (Table III). Unlike the haploid strains the homozygous deletion diploids had generation times significantly longer than the wild-type cells. This was particularly true for synthetic medium, where the deletion strains grew 10–12% slower than wild-type cells.

In addition to having longer generation times, the diploid deletion cells were also larger than their wild-type counterpart. Comparative histograms of cell volume distributions for early log phase growth cultures are shown in Fig. 4. The population distributions for the deletion strains were skewed towards larger cells. However, the deletion strains were not uniformly larger. The smallest cells in the culture were very similar in size for all three strains suggesting that the sizes of daughter cells at cell separation were the same.

Cell Cycle Parameters

The measurements of growth rates and cell volumes suggested that the histone gene deletions may have been affecting the cell division cycle. For example, a large defect in the S-period of the division cycle might be reflected in a small
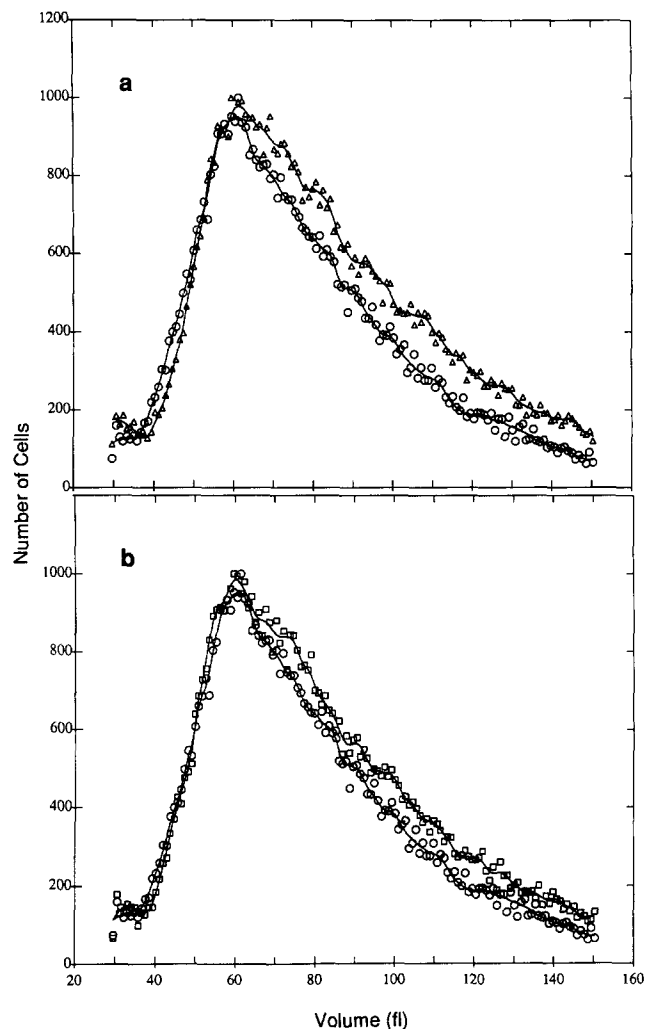

Figure 4. Cell volume profiles for the diploid histone deletion strains. The size distributions of cells from early logarithmic growth cultures were measured as described in Materials and Methods. The histogram of the number of cells in each channel is plotted against the cell volume of the channel. The results were separated into two panels for clarity. The distribution for the wild-type strain MSY167 is indicated by the open circles in both plots. The profile for the copy-I deletion diploid MSY164 is illustrated in the open triangles in *a* and the profile for the copy-II deletion diploid MSY165 is shown in the open squares in *b*.

increase in the average generation time and cell size for the H3-H4 deletion mutants (41). We reasoned that the decreased gene dosage of the H3 and H4 genes in the homozygous deletions might lead to an increase in the length of the S-phase of the cell cycle due to a defect in chromatin assembly. While we will show below that this is not the case, it was clearly of interest to obtain an estimate of the cell cycle timing in the deletion strains.

The cell cycle periods of the strains were examined by flow microfluorometry (44). For these experiments we used the set of diploid strains from cultures grown in YPD medium. We were unable to obtain reliable results from the haploid cells because of the low fluorescence signal from cells with G1 haploid DNA content. Diploid wild-type or histone gene deletion cells from early log phase cultures were harvested
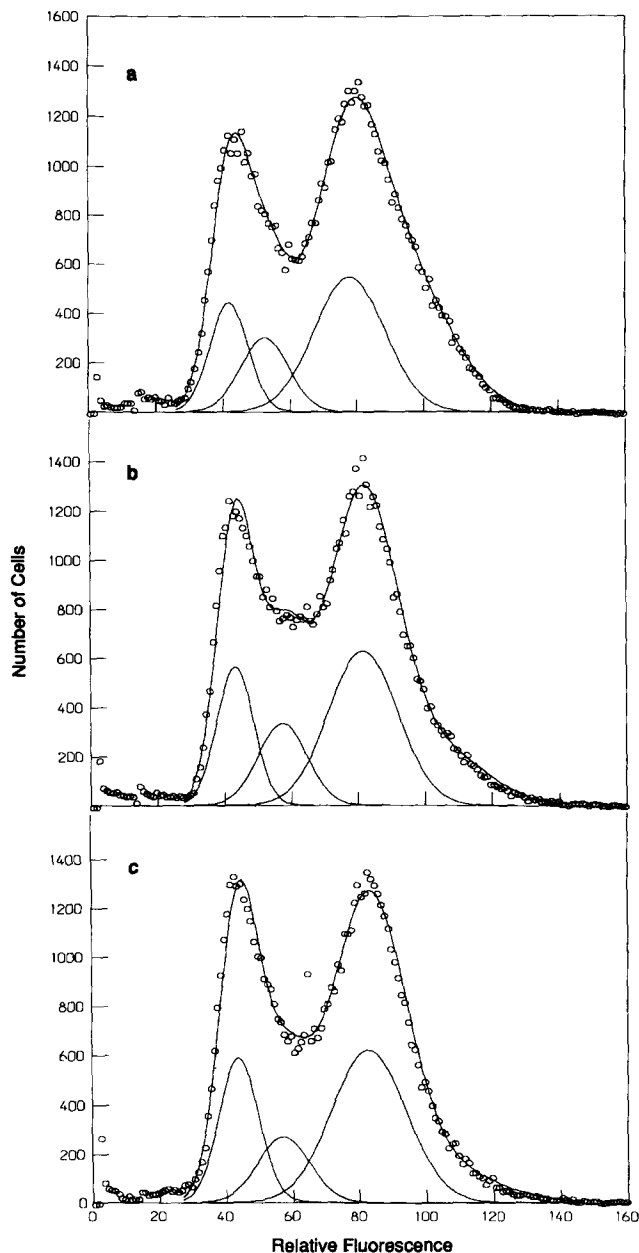
Figure 5. Flow microfluorometry of histone gene deletion strains. The fluorescence histograms for diploid strains from early logarithmic growth cultures were determined as described in Materials and Methods. The experimental fluorescence data are plotted in the open circles. The results of the model fitting calculations are shown as the solid lines through the experimental data. The results for the individual cell cycle periods are drawn at one-half scale under each curve. These represent the G1, S, and G2 period distributions, left to right, respectively. (a) MSY167 wild type. (b) MSY164 copy-I deletion. (c) MSY165 copy-II deletion.

and stained with mithramycin, a fluorescent DNA-binding dye (17, 43). The stained cells were analyzed for DNA content on an EPICS flow cytometer (Coulter Electronics, Inc., Hialeah, FL). Histograms of DNA content for the diploid strains are presented in Fig. 5. These histograms were decomposed into G1, S, and G2 populations as described in Materials and Methods and the results of these calculations are listed in Table IV.

Table IV. Cell Cycle Composition of Histone Deletion Strains

| Strain | Cell cycle period | Percentage of cells | Time | |
			Homogeneous cell model*	Mixed cell model‡
			min	min
MSY167	G1	21	14	29
Wild type	S	18	13	13
	G2	61	58	58
MSY164	G1	26	18	38
Copy-I	S	20	16	16
Deletion	G2	54	55	55
MSY165	G1	28	20	43
Copy-II	S	17	13	13
Deletion	G2	56	58	58

* Mother and daughter cells are asumed to have identical cell cycle compositions (44).
‡ Mother cells are assumed to proceed from cell separatoin directly into S phase. The G1 times shown are for the daughter cells in this model (44).

To a first approximation all three strains have similar cell cycle distributions. From this analysis we conclude that the H3-H4 gene deletion strains do not have a severe defect in a specific period of the division cycle. In particular, there was not a large increase in the proportion of cells in S-phase in the deletion strains. The major difference between the strains was an increase in the proportion of cells in the early periods of the division cycle in the deletion strains. From the measured generation times and the fraction of cells in each cell division cycle phase, the lengths of the periods can be estimated (44). The cell cycle analysis of *S. cerevisiae* is complicated by the fact that the two cells at cell separation are not equivalent; the larger "mother" may have a shorter G1 period than the smaller "daughter" cell (38). For this analysis we have adopted two limiting assumptions as proposed by Slater et al. (44). In the first model, the "homogeneous daughter model," it is assumed that all cells, both mother and daughter, have identical G1 periods. In the second model, the "mixed mother-daughter cell model," it is assumed that the mother cells do not have a G1 period at all but progress directly from cell separation into a new S period. The calculations for these two limiting assumptions are presented in Table IV. The length of the G2 period was similar for all three strains, ~57 min. Both deletion strains, however, had longer G1 periods, particularly for daughter cells in the mixed cell model calculations. Therefore, we conclude that the longer division times for the deletion strains are the result of an increase in the lengths of the early cell cycle periods, especially G1.

Mitotic Chromosome Stability

Mitotic chromosome transmission is sensitive to the stoichiometry of the histone gene loci. Overexpression of either an H3-H4 or H2A-H2B gene set from high copy number plasmids, or from a strong inducible promoter, can increase the frequency of chromosome loss by an order of magnitude (31). To test the effect of the H3-H4 deletions on chromosome stability, we used the color colony assay of Hieter et al. (23)

Table V. Mitotic Stability of Plasmid Minichromosome

Strain	Isolate	Cells scored	1:0 events	2:0 events	Total
			%	%	%
MSY167	1	2878	2.2	0.1	2.3
	2	2993	2.0	0.2	2.2
MSY164	1	3264	2.8	0.1	2.9
	2	3248	2.8	0.1	2.9
MSY165	1	1148	64.8	0.2	65
	2	1160	19.4	<0.1	19
	3	531	43.3	<0.2	43
	4	188	45.2	<0.5	45
	5	435	44.4	<0.2	44

using the plasmid pAB9SEG, a *CEN4*-ARS derivative of YRp14 (3). When a single copy of pAB9SEG is transformed into diploid cells homozygous for the ochre allele *ade2-101* the color phenotype of the colonies is pink on low adenine medium. The loss of the plasmid during mitosis can be scored as a pink/red half-sectored colony, the result of a 1:0 plasmid segregation event. Replication of the plasmid followed by nondisjunction gives a white/red half-sectored colony resulting from a 2:0 event. The plasmid pAB9SEG was used to transform each of the diploid strains and several individual transformants were selected and tested for the stability of the plasmid minichromosome. The results of these assays are present in Table V.

The wild-type diploid MSY167 and the copy-I deletion diploid MSY164 transformed normally and gave similar colony growth and morphology. Independent transformants were isolated and assayed for plasmid stability. When plated on low adenine indicator plates, <20% of the colonies were entirely red, indicating that most of the cells retained a copy of pAB9SEG at the time of plating. The frequencies of half-sectored 1:0 and 2:0 colonies were then scored with respect to total pink and sectored colonies. In the wild-type MSY167 the frequency of chromosome loss was ∼2% and the frequency of aberrant segregation was ∼0.2% generation. These results are similar to those reported previously (3, 23). The copy-I deletion strain showed a modest increase in the frequency of chromosome loss (1:0 events) approaching 3% per generation and a low frequency of 2:0 segregations.

The copy-II deletion strain had a more dramatic defect in mitotic minichromosome transmission. The plasmid pAB-9SEG transformed the diploid MSY165 at a normal frequency but the transformed colonies were smaller and slower growing than the wild-type or the copy-I deletion transformants and gave a mixture of colony sizes similar to defective ARS derivatives of YRp14CEN4 (3). Five independent transformants were isolated and assayed for plasmid stability. When grown on low adenine indicator plates over 75% of all the colonies were completely red. Scored as a fraction of the total pink and sectored colonies, the frequency of chromosome loss in these transformants varied from 19 to 65% per generation. Taken together these results indicate a high frequency of loss of the plasmid in the copy-II H3-H4 deletion diploid. Thus, these experiments support a model in which the ratios of the core histones are important for normal chromosome dynamics (31).

Discussion

The histone H3-H4 gene loci extend the list of nonallelic duplicated genes in *S. cerevisiae* that show a degree of functional redundancy. Some other examples include the histone H2A-H3B loci (27, 39), the yeast members of the *ras* oncogene family *RAS1* and *RAS2* (10, 11, 37), many of the ribosomal protein genes (1, 15), and the citrate synthase genes (26). For many duplicated genes in *S. cerevisiae* the two copies are not exact functional equivalents. For example, neither *ras* gene is required for cell viability; however, disruptions of *RAS2* result in detectable phenotypic defects whereas disruptions of *RAS1* do not. Similarly, deletions and gene disruptions at one of the duplicate H2A-H2B loci have little, if any, phenotypic effect, whereas mutations at the other set are expressed, although there are significant differences seen in the range and severity of these defects between laboratories (18, 27, 34, 39).

The two histone H3-H4 gene sets are more balanced in their functional expression. In haploid cells we did not detect any phenotypic expression of the deletions at either locus. However, the homozygous copy-II deletion diploid did show slight defects in generation time, G1-phase timing, and a pronounced decrease in plasmid minichromosome stability. Since the histones H3 and H4 encoded by the two sets of genes are identical, this distinction must reflect differences in the expression of the respective genes. The predominant importance of the copy-II locus is in agreement with the known expression and regulation of the H3 and H4 mRNAs (9). In wild-type cells, the steady-state levels of copy-II H3 and H4 mRNAs are five to seven times higher than the copy-I mRNAs. Furthermore, when the copy-II genes are deleted, the amounts of the copy-I H3 and H4 mRNAs do not increase. Thus, in copy-II deletion strains the levels of histone H3 and H4 mRNA are reduced to 15–20% of wild-type levels while in copy-I deletion strains the cells have 80–85% of normal mRNA levels. For cellular functions reflected in mitotic growth, mating, sporulation, germination, and cell cycle, the lower levels of histone H3 and H4 mRNA from copy-I genes must be sufficient to give a nearly wild-type phenotype. It is possible that histone H3 and H4 mRNA levels are normally at excess in wild-type cells and the reduced mRNA levels in the copy-II deletion are not rate limiting for histone H3 and H4 expression. In addition, regulation at the translational or posttranslational levels may compensate for the decrease in mRNA abundance. In any case, the minichromosome stability experiments suggest that cells cannot completely compensate for the loss of the copy-II mRNAs.

The increased frequency of plasmid minichromosome loss in the copy-II deletion diploid is in agreement with the landmark experiments of Meeks-Wagner and Hartwell (31) which demonstrated that the unbalanced expression of the core histone genes at high copy number can result in an increase in mitotic chromosome loss. The present experiments further strengthen the hypothesis that the stoichiometry of the core histones is important in mitotic chromosome transmission. First, it is now clear that both overexpresion and underexpression of histones H3 and H4 can result in increased chromosome loss. Second, it is likely that the critical stoichiometry is at the level of histone protein expression, and not at the simple level of gene dosage, since a significant increase in chromosome loss was seen with the copy-II deletion and

not the copy-I deletion. Finally, it is interesting to speculate that unbalanced histone stoichiometry results in an increase in plasmid DNA replication failure and thus chromosome loss. Since the plasmid minichromosome contains a single origin of replication, it would be particularly sensitive to replication failure. While the frequency of 1:0 events is the sum of replication failure plus all other sources of loss (23), such a model is at least consistent with our previous genetic studies of ARS function (3, 4) as well as many biochemical studies on the chromatin structure of viral replication origins and yeast ARS elements.

The effects of the H3-H4 gene deletions on the growth and cell cycle progression are particulary interesting. We expected that the increased generation times and larger mean cell volumes of the diploid deletion strains would be explained by an increase in the length of the S-period of the cells. Instead, the lengths of the cell cycle periods calculated from the generation times and flow cytometry data were roughly the same for the three strains. The major change in the H3-H4 deletion strains was an increase in the proportion of cells in the early periods of the cell cycle, particularly the G1 period. We can not rule out small changes in the S period of the deletion strains. The relatively low DNA content of *S. cerevisiae* limits the resolution that can be achieved by the flow cytometry experiments particularly for the S period. In addition, modeling the S phase as a single Gaussian function leads to a systematic underestimate of the proportion of S period cells. However, the distribution of cell sizes in exponential cultures is consistent with an increase in the length of the G1 period. The average volume of the smallest cells was the same for all three strains indicating that the growth period from bud emergence to cell separation is unaffected by the deletions. Therefore, it is likely that the growth defect in the deletion mutants is primarily a property of the G1 period. This result is distinct from that seen when histone gene expression is more stringently limited. For example, the complete repression of histone H2B results in the assembly of defective chromosomes and an irreversible block in mitosis (19). Similarly, deletion of the H2A1-H2B1 locus results in an increase in the length of the cell cycle outside of G1 (34).

There are several possible explanations for the cell cycle effects of the H3-H4 deletions. First, the increase in the G1 period, cell size, and generation time may reflect an increase in the aberrant mitotic transmission of natural chromosomes. In this model, daughter cells with a defective chromosome complement would continue growth for a period of time but leave the division cycle and eventually die. The net result would be an increase in the average cell volume of the population, and an apparent increase in the length of the G1 period and the population generation time. Experimentally the observed effects are small and cannot be resolved by viable cell counts. Experiments to measure the rates of natural chromosome loss in the deletion strains are in progress. Alternatively, it is possible that histone H3-H4 gene expression may be one of the parameters monitored by the regulatory mechanisms controlling cell proliferation and commitment to S-phase. Currently we have no direct evidence to position the defect early or late within the G1 period itself. Haploid deletion strains showed normal mating phermone arrest and mating type physiology, and the diploid deletions demonstrated normal commitment to sporulation. Taken together,

we reason that the H3-H4 gene deletions may result in a delay in the cell cycle late in the G1-period, after "start" and before DNA replication. A mutational analysis of the H3 and H4 genes remaining in the deletion strains may provide genetic evidence in support of this role.

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