Hir Proteins Are Required for Position-Dependent Gene Silencing in *Saccharomyces cerevisiae* in the Absence of Chromatin Assembly Factor I

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Chromatin assembly factor I (CAF-I) is a three-subunit histone-binding complex conserved from the yeast *Saccharomyces cerevisiae* to humans. Yeast cells lacking CAF-I (*cacΔ* mutants) have defects in heterochromatic gene silencing. In this study, we showed that deletion of *HIR* genes, which regulate histone gene expression, synergistically reduced gene silencing at telomeres and at the *HM* loci in *cacΔ* mutants, although *hirΔ* mutants had no silencing defects when CAF-I was intact. Therefore, Hir proteins are required for an alternative silencing pathway that becomes important in the absence of CAF-I. Because Hir proteins regulate expression of histone genes, we tested the effects of histone gene deletion and overexpression on telomeric silencing and found that alterations in histone H3 and H4 levels or in core histone stoichiometry reduced silencing in *cacΔ* mutants but not in wild-type cells. We therefore propose that Hir proteins contribute to silencing indirectly via regulation of histone synthesis. However, deletion of combinations of *CAC* and *HIR* genes also affected the growth rate and in some cases caused partial temperature sensitivity, suggesting that global aspects of chromosome function may be affected by the loss of members of both gene families.

Two molecules each of the core histones H2A, H2B, H3, and H4 tightly associate with 146 bp of DNA to form a nucleosome, the fundamental repeat structure of chromatin (38; for a review, see reference 73). Numerous genetic and biochemical studies have shown that core histones also contribute to gene activation (6, 16) and repression (18, 35) and have direct roles in position-dependent gene silencing (for reviews, see references 12 and 37). The latter point has been most clearly demonstrated in the budding yeast *Saccharomyces cerevisiae*, in which genes adjacent to telomeres and the mating type genes present at the silent *HM* loci are subject to position-dependent transcriptional repression. Mutations within the N-terminal residues of histones H3 and H4 disrupt this gene silencing (22, 41, 49, 71). Furthermore, these same N-terminal regions make direct contact with *SIR* gene products required for silencing in *S. cerevisiae* (14). Thus, chromatin structures responsible for gene silencing include nucleosomes as important components.

It is presently unknown how many proteins mediate assembly of histones on DNA in vivo or how such factors contribute to gene silencing. One factor involved in these processes is a three-subunit protein complex termed chromatin assembly factor I (CAF-I). CAF-I was initially purified from human somatic cell nuclear extracts on the basis of its ability to assemble histone octamers during DNA replication in vitro (62, 66; for reviews, see references 26 and 32). CAF-I-like activities have also been detected in extracts of *Drosophila* and *Xenopus* embryos (25). Purification of yeast CAF-I led to identification of the genes encoding the three yeast CAF-I subunits, each of which is homologous to its human counterpart (28). These genes have been termed *CAC1*, *CAC2*, and *CAC3* (for chromosome assembly complex). Deletion of any of the three *CAC*

genes results in decreased telomeric gene silencing (9, 28). *cacΔ* mutants display decreased stability of the transcriptionally silent state at both telomeres and the silent *HML* locus (8, 42). However, *cacΔ* mutants have no known growth defects. These data indicate that yeast CAF-I contributes to position-dependent gene silencing but is not the only cellular factor responsible for chromatin assembly.

Loss of *SIR2*, *SIR3*, or *SIR4* gene function in *S. cerevisiae* completely abolishes gene silencing at both *HM* loci and telomeres (1, 55). These genes encode proteins that are structural components of silenced heterochromatin (15, 67) and are not known to be involved in the assembly of histones on DNA. Indeed, mutants specifically defective in histone deposition have not been described. There could be several reasons for this. Formation of nucleosomes from newly synthesized histones during DNA replication is an essential process because progression through S phase of the cell cycle in the absence of histone H2B or H4 synthesis causes lethality (13, 30). However, if the process of chromatin formation were performed by multiple, partially redundant factors, recovery of mutations in this process would be difficult in standard genetic screens because of the weak phenotypes of single mutants. In this case, disruption of multiple factors would be required to observe strong phenotypes resulting from chromatin malformation or malfunction.

We describe here a novel phenotype of *cacΔ* mutants: the residual gene silencing in these cells is sensitive to mutations in the *HIR1*, *HIR2*, and *HIR3* genes and to changes in histone gene dosage. The three *HIR*-encoded proteins act to restrict transcription of three of the four histone gene pairs to the *G₁/S* phase transition of the yeast cell cycle and to regulate the *HTA1-HTB1* locus in response to altered levels of the gene products of this locus, histones H2A and H2B (43, 48, 60, 65). The *HIR* gene products regulate the histone *HTA1-HTB1* promoter through a negative *cis*-acting site, although the Hir proteins themselves do not appear to directly contact DNA. *hirΔ*

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mutants display a *Hir*[−] phenotype: *HTA1-HTB1* transcription is not repressed (i) outside of the G₁/S phase; (ii) when cells are treated with hydroxyurea (HU), which inhibits DNA synthesis and causes accumulation of unassembled histone proteins; or (iii) when the *HTA* and *HTB* genes are present on high-copy-number plasmids. Although *hir*Δ mutants had no defects in gene silencing when CAF-I was intact, we observed a synergistic loss of telomeric and *HM* gene silencing in *cac*Δ *hir*Δ double mutants. These data suggest that pathways responsible for formation of heterochromatin in the absence of CAF-I are easily perturbed by changes in histone levels or histone stoichiometry. Furthermore, we provide evidence that the *Hir* proteins also have a role in cooperating with CAF-I to ensure proper cell growth and viability.

MATERIALS AND METHODS

Plasmids. To make the *cac3Δ::LEU2* deletion allele, a *HindIII*-*Bam*HI fragment of pJJ283 (23) containing the *LEU2* gene was inserted into *Clal*- and *Bgl*II-cut pPK96 (28) to generate pPK112. pPK112 was digested with *Apa*I and *Sac*I prior to transformation.

To make the *cac1Δ::hisG-URA3-hisG* deletion allele, a 5.4-kb *Bam*HI-*Xba*I fragment containing the *URA3* and kanamycin resistance (*kan*) genes flanked by direct repeats of bacterial *hisG* DNA was inserted into *Bgl*II- and *Nhe*I-digested pPK98 (28) to create pPK102. pPK102 was digested with *Bam*HI for transformation.

To make the *cac2Δ::hisG-URA3-hisG* deletion allele, a 5.4-kb *Bam*HI-*Bgl*II fragment containing the *hisG-URA3-kan-hisG* DNA was inserted into *Sna*BI- and *Sph*I-digested, Klenow polymerase-treated pPK55 (28) to create pPK101. pPK101 was digested with *Eco*RI and *Kpn*I prior to transformation.

To make pPK118 [YEplac351-(HHT1-HHF1)], the 5.5-kb *HindIII*-*Bam*HI fragment from pCC67 (3) was inserted into *HindIII*- and *Bam*HI-digested YEplac351. To make pPK119 [YEplac181-(HHT2-HHF2)], the 2.7-kb *Sma*I-*Eco*RI fragment from pCC66 (3) was inserted into *Sma*I- and *Eco*RI-digested YEplac181. To make pPK120 [YEplac181-(HTA2-HTB2)], the 3.55-kb *Sma*I-*Eco*RI fragment from pCC223 (3) was inserted into *Bam*HI- and *Eco*RI-digested YEplac181. To make pPK128 [YEplac351-(HHT1-HHF1)-(HTA1-HTB1)], the 6.4-kb *Bam*HI fragment of pCC67 (3) was inserted into *Bam*HI-digested pPK118.

To make the *HTA1*Δ-neg transplacement fragment, a 1.1-kb *HindIII* fragment from the *HTA1-HTB1* promoter, containing a 54-bp deletion of the *Hir*-responsive negative site marked by a *Xho*I site (47), was inserted into a pUC18-based plasmid that contained a *HindIII*-*Sac*I insert carrying the 3' end of the *HTB1* gene and the *HIS3* gene inserted as a *Bam*HI fragment. The resulting plasmid, pMA100, was digested with *Sal*I and *Eco*RI to liberate an ~4.0-kb fragment that contained the entire *HTA1-HTB1* promoter with the negative site deleted, the entire *HTB1* gene and 3'-flanking sequences, and the *HIS3* gene.

Yeast strains. The genotypes of yeast strains used in this study are shown in Table 1. All strains used were derived from strain W303 (70) by transformation or by crosses with other strains in this background, with the exception of the *spz21Δ::HIS3* allele, which was backcrossed from strain GNX193-1B (44) to W303 strains six times prior to the construction of the strains used here. Previously described deletion alleles include the following: *cac1Δ::LEU2*, *cac2Δ::TRP1*, *cac3Δ::URA3*, *cac3Δ::hisG-URA3-kan-hisG*, and *URA3-VIII* (28); *hir1Δ::HIS3* and *hir3Δ::HIS3* (53); *sir1Δ::LEU2* (21); (*hht1-hhf1*)Δ::*LEU2* (plasmid pUK192 [39]); (*hht2-hhf2*)Δ::*HIS3* (pUK431 [39]); *hhf2Δ::LEU2* (pPK21 [29]); (*hta2-htb2*)Δ::*TRP1* (pJH21 [16]); and *hmlΔ::LEU2* (pJH455 [76]).

Gene disruptions were performed by lithium acetate transformation of a wild-type diploid as described elsewhere (24). Selected colonies were purified twice after transformation and then sporulated. Correct gene disruptions were confirmed in all cases by DNA hybridization. The *HTA1*Δ-neg-*HIS3* strains were further checked to ensure that they had a *Hir*[−] phenotype (48): total RNA isolated from log-phase cells treated with 0.2 M HU for 30 min prior to harvest was analyzed by Northern blot hybridization to show that the *HTA1* mRNA was not down regulated (data not shown).

Genetic procedures and media. Standard procedures were used for genetic crosses and tetrad analysis. Standard yeast media used for crosses and for scoring genetic marker segregations were as described elsewhere (24). YPAD is yeast extract-peptone-dextrose (YPD) medium supplemented with adenine at 50 mg/liter. 5-Fluoro-orotic acid (FOA) was added in all cases to synthetic media at a concentration of 1 mg/ml.

Viability and growth assays. Viability tests were conducted by plating serial dilutions of cells growing exponentially at 30°C in YPD broth onto prewarmed YPD plates and incubating at 30 and 37°C. Samples (80 μl fixed in phosphate-buffered saline-3.7% formaldehyde) were also counted with a hemocytometer immediately before duplicate platings were performed to determine total cell numbers. Generation times were determined in YPD medium at 30 or 37°C by following the absorbance at 660 nm; for several *cac*Δ *hir*Δ double mutants, generation times were determined by counting cell numbers with a hemocytom-

eter. Because the *trp1-1* allele present in the W303 strain background causes slow growth at 16°C, we performed control experiments to show that *cac*Δ *hir*Δ double mutants had growth defects at this temperature that were independent of the status of the *TRP1* gene (data not shown).

Telomeric silencing assays. To measure telomeric silencing, the *URA3* gene inserted next to the chromosome VIII telomere was used as previously described (28); originally described in reference 11). We found that equivalent results were obtained by using log-phase cells (*A*₆₀₀ ~0.6) from liquid medium or cells taken from plates and adjusted to an *A*₆₀₀ of ~0.6 (see, e.g., reference 57). To assess quantitatively the proportion of silenced cells in a population, cells were plated on synthetic medium with or without FOA (either complete or selective for plasmids for the data in Table 4), and the number of FOA-resistant colonies per viable cell plated was determined after 8 days of incubation at 30°C. Small FOA-resistant microcolonies formed by the *cac*Δ strains were counted under a dissecting microscope. Colonies on synthetic complete (or selective)-medium plates were counted after 3 days to assess the number of viable plated cells. To correct for variations in the potency of the FOA in different batches of plates, the fraction of FOA-resistant cells was normalized to that of the wild-type strain for each repetition of the experiment. In experiments using strains carrying plasmids, duplicate transformants of each strain tested gave the same results. In some cases, visual, semiquantitative estimates of telomeric silencing were obtained by spotting 5-μl quantities of 10-fold serial dilutions of cells onto medium with or without FOA; however, data from these spot tests were not used to obtain the numerical values presented.

Quantitative mating assays. Quantitative assays were performed as described elsewhere (7), using strains 216 (*MATα his1*) and 217 (*MATα his1*) as the testers. Patch mating tests (see Fig. 3C) were performed by growing cell patches overnight on YPD medium at 30°C and then replica plating the patches either onto synthetic dextrose agar plates onto which had been spread a fresh culture of the appropriate mating tester or onto YPD plates as a control for cell growth. Patches were photographed after 2 days of growth at 30°C.

Dam methylase accessibility assays. These assays were performed essentially as described elsewhere (33), except that the enzyme *Dpn*II (New England Biolabs) was used instead of *Mbo*I. In this case, DNA was first digested with *Hind*III and then reaction mixtures were adjusted with the buffer recommended by the manufacturer for digestion with *Dpn*II.

RESULTS

The *CAC2/HIR1* subfamily of WD repeat proteins. The *CAC2* and *CAC3* genes encode the smaller two subunits of yeast CAF-I (28). *Cac2p* and *Cac3p* contain WD repeat motifs found in many eukaryotic proteins (45). *Cac3p* (previously described in the literature as *Msi1p* [19, 28, 56]) is a member of the histone-binding p48 subfamily of WD proteins which includes the p48 subunit of human CAF-I, the yeast histone acetyltransferase-associated protein *Hat2p*, and the human histone acetyltransferase-associated protein *p46* (28, 50, 74, 75). All members of the p48 subfamily share conserved residues outside the canonical WD repeat signature and are members of at least one multisubunit protein complex that either binds or modifies histone proteins (28, 50, 69, 72, 74, 75, 79). *Cac2p* is not a member of the p48 subfamily. However, examination of database entries showed that the yeast protein most similar to *Cac2p* is *Hir1p*, which contains WD motifs in its N-terminal half (34, 60). The yeast *Cac2p* and *Hir1p* proteins both have conserved human homologs, the CAF-I p60 subunit and the *HIRA* protein, respectively (27, 34). Comparison of *CAC2*, *HIR1*, and their human homologs showed that they encode a distinct subfamily of WD proteins (Fig. 1). Each of the seven WD repeats in these proteins includes conserved residues other than those that define a generic WD motif consensus, and each repeat contains distinctly conserved residues not shared by the other six repeats (Fig. 1). Furthermore, the N-to-C-terminal order of the WD repeats in these four proteins is conserved.

Regulation of histone gene promoters by *Hir* proteins is thought to involve the formation of repressive chromatin structures by the histone-binding protein *Hir2p* (3a), suggesting that the homology shared by *CAC2* and *HIR1* may reflect the presence of a conserved domain involved in chromatin assembly. One phenotypic consequence of defects in chromatin assembly is the reduction of heterochromatic gene silencing (28). We

TABLE 1. *S. cerevisiae* strains used in this study

Strain ^a	Genotype	Reference or source
W303-1A	<i>MATa</i>	70
W303Δ1H	<i>MATa hir1Δ::HIS3</i>	53
W303Δ2	<i>MATa hir2Δ::URA3</i>	60
W303Δ1Δ2(FOA)	<i>MATa hir1Δ::HIS3 hir2Δ::ura3</i> (FOA) [†]	58
YB 0152	<i>MATa cac2Δ::TRP1</i>	28
AJL387-5a Δsir2	<i>MATa rad1Δ::LEU2 sir2Δ::TRP1 LYS2::dam/HIS3::lys2 URA3-VIIL</i>	33
JRY4470	<i>MATa sir2Δ::LEU2 ura3::LEU2 TRP1-URA3-VIIL</i>	J. Rine (University of California, Berkeley)
PKY028	<i>MATa</i>	28
PKY031	<i>MATa cac2Δ::hisG-URA3-kan-hisG</i>	This work
PKY034	<i>MATa cac3Δ::hisG-URA3-kan-hisG</i>	This work
PKY035	<i>MATa cac1Δ::hisG-URA3-kan-hisG</i>	This work
PKY090	<i>MATa URA3-VIIL</i>	28
PKY102	<i>MATα cac1Δ::LEU2 hir1Δ::HIS3</i>	This work
PKY103	<i>MATa cac2Δ::TRP1 hir1Δ::HIS3</i>	This work
PKY106	<i>MATa cac1Δ::LEU2 URA3-VIIL</i>	28
PKY107	<i>MATa cac2Δ::TRP1 URA3-VIIL</i>	28
PKY110	<i>MATα cac1Δ::LEU2 hir1Δ::HIS3 hir2Δ::URA3</i>	This work
PKY111	<i>MATa cac2Δ::TRP1 hir1Δ::HIS3 hir2Δ::URA3</i>	This work
PKY112	<i>MATa cac1Δ::LEU2 cac2Δ::TRP1 hir1Δ::HIS3 hir2Δ::URA3</i>	This work
PKY117	<i>MATa hir1Δ::HIS3 URA3-VIIL</i>	This work
PKY132	<i>MATα cac3Δ::URA3 hir1Δ::HIS3</i>	This work
PKY136	<i>MATa cac1Δ::LEU2 hir2Δ::URA3</i>	This work
PKY137	<i>MATα cac2Δ::TRP1 hir2Δ::URA3</i>	This work
PKY149	<i>MATa sir1Δ::LEU2 cac2Δ::TRP1</i>	This work
PKY264	<i>MATa cac2Δ::TRP1 hir1Δ::HIS3 URA3-VIIL</i>	This work
PKY268	<i>MATa HMR⁺::ADE2</i>	This work
PKY269	<i>MATa HMR⁺::ADE2 cac1Δ::LEU2</i>	This work
PKY299	<i>MATa cac1Δ::LEU2 LYS2::dam/HIS3::lys2 URA3-VIIL</i>	This work
PKY300	<i>MATa LYS2::dam/HIS3::lys2 URA3-VIIL</i>	This work
PKY302	<i>MATa cac1Δ::LEU2 hir1Δ::HIS3 URA3-VIIL</i>	This work
PKY305	<i>MATa cac2Δ::TRP1 LYS2::dam/HIS3::lys2 URA3-VIIL</i>	This work
PKY310	<i>MATa cac2Δ::TRP1 hir1Δ::HIS3 LYS2::dam/HIS3::lys2 URA3-VIIL</i>	This work
PKY311	<i>MATa hir1Δ::HIS3 LYS2::dam/HIS3::lys2 URA3-VIIL</i>	This work
PKY328	<i>MATa hir3Δ::HIS3</i>	This work
PKY329	<i>MATa hir1Δ::HIS3 spt21Δ::HIS3 URA3-VIIL</i>	This work
PKY332	<i>MATa cac3Δ::LEU2 spt21Δ::HIS3 URA3-VIIL</i>	This work
PKY352	<i>MATa hir3Δ::HIS3 URA3-VIIL</i>	This work
PKY353	<i>MATa cac2Δ::TRP1 hir3Δ::HIS3 URA3-VIIL</i>	This work
PKY354	<i>MATa hir3Δ::HIS3 spt21Δ::HIS3 URA3-VIIL</i>	This work
PKY360	<i>MATa cac1Δ::LEU2 spt21Δ::HIS3 URA3-VIIL</i>	This work
PKY361	<i>MATa spt21Δ::HIS3 URA3-VIIL</i>	This work
PKY365	<i>MATa cac3Δ::LEU2 hir3Δ::HIS3 URA3-VIIL</i>	This work
PKY375	<i>MATa HMR⁺::ADE2 cac1Δ::LEU2 hir1Δ::HIS3</i>	This work
PKY377	<i>MATa cac2Δ::TRP1 spt21Δ::HIS3 URA3-VIIL</i>	This work
PKY378	<i>MATa sir1Δ::LEU2</i>	This work
PKY380	<i>MATa sir1Δ::LEU2 hir3Δ::HIS3</i>	This work
PKY382	<i>MATa cac2Δ::TRP1 hir3Δ::HIS3</i>	This work
PKY384	<i>MATa sir1Δ::LEU2 cac2Δ::TRP1 hir3Δ::HIS3</i>	This work
PKY408	<i>MATa (hht1-hhf1)Δ::LEU2 URA3-VIIL</i>	This work
PKY409	<i>MATa cac2Δ::TRP1 (hht1-hhf1)Δ::LEU2 URA3-VIIL</i>	This work
PKY410	<i>MATa (hht2-hhf2)Δ::HIS3 URA3-VIIL</i>	This work
PKY411	<i>MATa cac2Δ::TRP1 (hht2-hhf2)Δ::HIS3 URA3-VIIL</i>	This work
PKY412	<i>MATa hhf2Δ::LEU2 URA3-VIIL</i>	This work
PKY413	<i>MATa cac2Δ::TRP1 hhf2Δ::LEU2 URA3-VIIL</i>	This work
PKY499	<i>MATa (hta2-htb2)Δ::TRP1 URA3-VIIL</i>	This work
PKY500	<i>MATa cac1Δ::LEU2 (hta2-htb2)Δ::TRP1 URA3-VIIL</i>	This work
PKY512	<i>MATa cac1Δ::hisG-URA3-kan-hisG cac2Δ::TRP1</i>	This work
PKY581	<i>MATa HTA1Δneg::HIS3 URA3-VIIL</i>	This work
PKY582	<i>MATa cac1Δ::LEU2 HTA1Δneg::HIS3 URA3-VIIL</i>	This work
PKY730	<i>MATa hm1Δ::LEU2 sir1Δ::LEU2 cac2Δ::TRP1 hir3Δ::HIS3</i>	This work

^a All strains are derived from the W303 (70) genetic background and have the genotype *leu2-3,112 ura3-1 his3-11,15 trp1-1 ade2-1 can1-100*.

expect that assembly of chromatin is accomplished by a large number of functionally overlapping factors since *cacΔ* mutants display only a partial loss of telomeric silencing. We therefore tested whether deletion of both *CAC* and *HIR* genes in the

same cell would result in more-severe phenotypes with respect to heterochromatic gene silencing and histone gene regulation.

Growth defects in *cacΔ hirΔ* mutants. Loss of single *CAC* or *HIR* genes does not result in growth defects, nor does loss of

FIG. 1. The *CAC2/HIR1* subfamily of WD repeat proteins. The yeast *CAC2* gene (28), the human CAF-I p60 gene (27), the human *HIRA* gene (34), and the yeast *HIR1* gene (34, 60) were aligned by using the PILEUP program (Genetics Computer Group, Madison, Wis.). Each protein sequence is shown starting from the initiator methionine through the seven WD motifs depicted on each line. Amino acids identical in at least three proteins are shadowed in black; conservative changes are underlined. A WD motif consensus from a large number of proteins is illustrated between repeats 3 and 4 (45), with shadowed H's representing hydrophobic residues and DPGN representing a region that often includes those amino acids.

multiple members within the same group (9, 28, 60) (Fig. 2). However, most *cacΔ hirΔ* double mutants grew somewhat more slowly than wild-type or single-mutant cells at 30°C, as reflected by decreased colony sizes on plates; this phenotype was exacerbated at low (16°C) (data not shown) and high (37°C) (Fig. 2A) temperatures, although there was no specific morphological defect (e.g., cell cycle arrest phenotype) associated with growth at 37°C. These colony phenotypes arose only when deletions of *CAC* and *HIR* genes were combined, and they were most pronounced in *cac2Δ hir2Δ* double mutants and in triple mutants with deletions of both *HIR1* and *HIR2*, for which growth defects were noted at both 30 and 37°C (Fig. 2B). *cacΔ hirΔ* double-mutant cells also had longer generation times than *cacΔ* or *hirΔ* single-mutant cells in liquid YPD medium. For example, *cac2Δ* or *hir2Δ* single-mutant strains doubled every 90 to 100 min at both 30 and 37°C, whereas *cac2Δ hir2Δ* double mutants had a 125-min generation time at 30°C and a 195-min generation time at 37°C (Table 2). A *cac2Δ hir1Δ hir2Δ* triple mutant had an even longer generation time, doubling every 225 min at 30°C. Also, *cacΔ hirΔ* colonies were smaller after tetrad dissection, including those double mutants that do not have significantly decreased colony sizes or increased doubling times at 30°C in liquid culture (e.g., *cac1Δ hir1Δ* mutants [Fig. 2C]). In contrast, small tetrad colony phenotypes were never observed on deletion of multiple members of either the *CAC* or *HIR* gene family (data not shown).

The slower growth rates of *cacΔ hirΔ* double mutants at 37°C did not generally result from cell inviability because, with one exception, the double mutants were equally viable when plated on YPD medium at 30 and 37°C (Table 2). The exception was the *cac2Δ hir2Δ* double mutant, which showed a 4- to 10-fold

loss of viability at 37°C but not at 30°C. *cac2Δ hir1Δ hir2Δ* triple mutants and *cac1Δ cac2Δ hir1Δ hir2Δ* quadruple mutants, however, exhibited not only very reduced colony sizes but also reduced viability at 37°C (Fig. 2B). For example, >99% of *cac2Δ hir1Δ hir2Δ* cells were inviable when plated at 37°C, although the triple mutant showed no loss of viability when plated at 30°C (Table 2).

The synthetic growth phenotypes could occur because *cacΔ hirΔ* double mutants are more severely defective in histone synthesis and/or chromatin formation than single mutants. To test the first possibility, we examined whether *cacΔ* mutants displayed appropriate transcriptional regulation of the histone H2A1-encoding *HTA1* gene (60). Using an *HTA1-lacZ* reporter gene, we found that transcription from the *HTA1* promoter was unaltered from that of the wild type in both single and double *cacΔ* mutant cells: there was no observed effect on the overall levels of *HTA1-lacZ* transcripts or on the ability of the reporter gene to be repressed when DNA synthesis was blocked with HU or when extra copies of genes encoding histones H2A and H2B were present (data not shown and references 43 and 60). In addition, the *HTA1* reporter gene was derepressed to the same extent in *hirΔ* single mutants and in *cacΔ hirΔ* double mutants (data not shown). Therefore, CAF-I does not appear to regulate *HTA1* transcription.

Synergistic effects of *cacΔ* and *hirΔ* mutations on telomeric gene silencing. To test whether the growth defects of *cacΔ hirΔ* double mutants were due to defective chromatin formation, we first analyzed telomeric chromatin structure and function. In *S. cerevisiae*, telomere-proximal genes are subject to position-dependent but gene-independent silencing, termed the telomeric position effect (TPE) (11). TPE can be quantitatively

FIG. 2. Growth defects of *cacΔ hirΔ* mutants. Cultures were streaked onto YPD plates for distribution into single colonies, and the plates were incubated for 3 to 5 days. (A) *cacΔ hirΔ* strains grown at 37°C; (B) triple and quadruple *cacΔ hirΔ* deletion strains grown at 30 and 37°C. Strains used were W303 (*HIR⁺ CAC⁺*), W303Δ1 (*hir1Δ*), W303Δ2 (*hir2Δ*), W303Δ1Δ2(*FOA*) (*hir1Δ hir2Δ*), PKY035 (*cac1Δ*), PKY031 (*cac2Δ*), PKY034 (*cac3Δ*), PKY102 (*cac1Δ hir1Δ*), PKY103 (*cac2Δ hir1Δ*), PKY132 (*cac3Δ hir1Δ*), PKY136 (*cac1Δ hir2Δ*), PKY137 (*cac2Δ hir2Δ*), PKY103 (*cac3Δ hir2Δ*), PKY110 (*cac1Δ hir1Δ hir2Δ*), PKY111 (*cac2Δ hir1Δ hir2Δ*), and PKY112 (*cac1Δ cac2Δ hir1Δ hir2Δ*). (C) Slow growth of *cac1Δ hir1Δ* spores after germination. Strain PKY102 (*MATα cac1Δ::LEUZ hir1Δ::HIS3*) was mated to PKY090 (*MATα URA3-VIII*). The resulting diploid was sporulated, and tetrads were dissected. The viable progeny, shown here after 3 days of growth on YPAD medium at 30°C, are of two sizes. Scoring of markers revealed that all of the smaller progeny were *His⁺ Leu⁺*, indicating that they were *cac1Δ hir1Δ* double mutants (data not shown). Note that the number of complete tetrads with a 3:1 ratio of large to small colonies is below the expected 2:3 frequency for tetratypes; this is presumably because of the proximity of *CAC1* to the chromosome XVI centromere.

assayed by placing the *URA3* gene adjacent to a telomere; the fraction of cells in a population that are resistant to the drug FOA, a metabolic poison for *Ura⁺* cells, represents the level of silencing of the *URA3* gene. In *cacΔ* mutants, TPE is reduced but not abolished (9, 28). We quantitated the levels of TPE in *cacΔ* mutants, *hirΔ* mutants, and *cacΔ hirΔ* double mutants. *hir1Δ* mutants had no defects in TPE; in fact, there was a slight increase in the TPE in these strains (Fig. 3A). *hir1Δ* mutants were indistinguishable from *hir2Δ* or *hir3Δ* mutants in this assay (data not shown). In contrast, *cacΔ hirΔ* double mutants had TPE levels approximately 3 orders of magnitude below the levels observed for the partially derepressed *cacΔ* mutants. These synergies were observed in multiple *cacΔ hirΔ* double-mutant combinations: deletion of *cac1Δ* or *cac2Δ* in combination with either *hir1Δ*, *hir2Δ*, or *hir3Δ* gave similar results, with the fraction of FOA-resistant colonies being in the range of 10^{-4} to 10^{-5} relative to wild-type cells (Fig. 3A and data not shown). *cac3Δ* mutants had a weaker TPE defect than *cac1Δ* or *cac2Δ* mutants (28), and *cac3Δ hirΔ* double mutants were also slightly less derepressed than other *cacΔ hirΔ* combinations (data not shown).

The *HIR1*, *HIR2*, and *HIR3* genes do not encode the only

TABLE 2. Generation times and viabilities of yeast strains at 30 and 37°C^a

Strain	Relevant genotype	Generation time (min, 30°C)	% Viability at 37°C ^b
W303-1A	Wild type	95	100
W303Δ1	<i>hir1Δ</i>	90	100
W303Δ2	<i>hir2Δ</i>	90	100
W303Δ1Δ2(FOA)	<i>hir1Δ hir2Δ</i>	105	100
PKY080	<i>cac1Δ</i>	90	100
PKY076	<i>cac2Δ</i>	90	100
PKY512	<i>cac1Δ cac2Δ</i>	105	100
PKY102	<i>cac1Δ hir1Δ</i>	95	100
PKY103	<i>cac2Δ hir1Δ</i>	110	100
PKY136	<i>cac1Δ hir2Δ</i>	125	100
PKY137	<i>cac2Δ hir2Δ</i>	125	18
PKY110	<i>cac1Δ hir1Δ hir2Δ</i>	130	56
PKY111	<i>cac2Δ hir1Δ hir2Δ</i>	225	<1.0

^a At 37°C, *cac2Δ hir2Δ* and *cac1Δ hir1Δ hir2Δ* strains produced colonies with two classes of colony sizes: the majority of colonies were small and a minority were large after 3 days. At 30°C, all of the colonies were large.

^b Percent viability values are the averages of data from two independent experiments, except for strain W303Δ1Δ2(FOA), which was assayed once. All strains tested were 100% viable at 30°C.

regulators of histone gene transcription. Mutations of several genes alter the site of transcription initiation at gene promoters made nonfunctional by Ty transposon insertions (for a review, see reference 77). Mutants lacking these genes (termed *SPT* [for suppressor of Ty] genes) thus have an *Spt*[−] phenotype in which a functional transcription start site is restored to gene promoters with Ty insertions. A subset of *spt* mutants also have a *Hir*[−] phenotype because they are unable to repress *HTA1* transcription upon treatment with HU; conversely, *hirΔ* mutations also alter Ty transcription and thus cause *Spt*[−] phenotypes (59, 64). *SPT* genes that are required for a normal *Hir*⁺ phenotype include *SPT1*, which is identical to *HIR2* (59); *SPT11* and *SPT12*, encoding histones H2A and H2B (3); and *SPT10* and *SPT21*, which are required for transcription of the *HTA2-HTB2* and *HHT2-HHF2* histone gene pairs (5).

To test whether other genes that misregulate histone gene expression also affect the TPE, we measured telomeric silencing in *spt21Δ*, *cacΔ spt21Δ*, and *hirΔ spt21Δ* strains (Fig. 3A and data not shown). In all combinations, the *spt21Δ* deletion behaved like a *hirΔ* deletion with respect to telomeric gene silencing. *spt21Δ* cells had nearly wild-type levels of TPE (the approximately twofold reduction in TPE could be due to the fact that this mutation is pleiotropic, affecting expression at a large number of loci [44]). Combining *spt21Δ* with a *hir1Δ* or *hir3Δ* deletion did not result in a synergistic change in the TPE (Fig. 3A and data not shown), as observed for combinations of *hirΔ* deletions (data not shown). In contrast, *cacΔ spt21Δ* double mutants were as severely reduced for TPE as were *cacΔ hirΔ* double mutants (Fig. 3A and data not shown). Thus, these data extend our observation that mutations that cause misregu-

lation of histone synthesis also cause a dramatic reduction of telomeric silencing in *cacΔ* mutants.

Synergistic effects of *cacΔ* and *hirΔ* mutations on *HM* gene silencing. Position-dependent transcriptional repression at the *HML* and *HMR* loci of *S. cerevisiae* ensures that only the *a* or *α* genes at the *MAT* locus are expressed; defects in silencing at *HML* or *HMR* cause coexpression of *a* and *α* genes in the same cell, resulting in a reduced mating efficiency (for a review, see reference 37). Although *cacΔ* mutants mate as efficiently as wild-type cells (9, 28) (Table 3), we asked whether combinations of *cacΔ* and *hirΔ* mutations cause synergistic silencing defects at *HM* loci. We quantitated mating of *MATa* strains as an assay for silencing of the *α* genes at *HML*, and we measured mating of isogenic *MATα* strains as an assay for silencing of the *a* genes at *HMR*. As previously observed, *cacΔ* or *hirΔ* gene deletions by themselves did not confer a mating defect (Table 3). However, in *MATa cacΔ hirΔ* double mutants (e.g., *cac2Δ hir3Δ* [Table 3]), approximately a 10-fold reduction in mating was observed, indicative of *HML* derepression. This demonstrates that the synergistic loss of position-dependent gene silencing in *cacΔ hirΔ* mutants is not limited to telomere-proximal genes.

Silencing at *HMR* is stronger than that at *HML* because of the more highly redundant nature of the *cis*-acting silencer *HMR-E* (37). For example, *cac2Δ hir3Δ* mutants did not display an *HMR* silencing defect as assayed by mating (Table 3). However, more-sensitive assays for reduction in silencing at *HMR*, which rely on the presence of mutations within the silencers or on different reporter genes, have been developed. In one case, it was found that replacing the *a* genes normally present at *HMR* with the *ADE2* gene allows for very sensitive detection of changes in silencing by a colony color assay (68). Our strains carry an *ade2-1* mutation at the *ADE2* locus, which causes red colony color on adenine-limited media; the *HMR::ADE2* allele is largely repressed, rendering cells pink. However, *cis*- or *trans*-acting mutations that reduce silencing lead to pink- or white-sectored or entirely white colonies because of increased expression of the *ADE2* gene. We found that *CAC* gene deletions gave strains carrying the *HMR::ADE2* allele a mottled pink and white appearance, in which a large variety of shades of pink are evident in a population (shown as a population of mixed dark and light colonies in a black-and-white photograph [Fig. 3B]). This suggests that cells are rapidly switching between the silenced and nonsilenced states at *HMR::ADE2* in *cacΔ* mutants, consistent with recent data examining switching at telomeric and *HML* loci (8, 42). In contrast, *hirΔ* mutants showed the same uniformly pink colony color as wild-type cells (data not shown). *cacΔ hirΔ HMR::ADE2* mutants appeared white (Fig. 3B), suggesting that the two mutations act synergistically at *HMR* to derepress transcription, as we had observed at telomeres and *HML*.

The *SIR1-4* (for silent information regular) genes are important for efficient silencing at the *HM* loci (for a review, see reference 37). Deletion of either *SIR2*, *SIR3*, or *SIR4* results in

FIG. 3. Deletion of both *CAC* and *HIR* genes synergistically reduces silencing of a telomere-proximal *URA3* gene, the *ADE2* gene at *HMR*, and the *a* genes at the natural *HML* locus. (A) Quantitation of silencing in representative strains. PKY090 (*CAC*⁺), PKY106 (*cac1Δ*), PKY117 (*hir1Δ*), PKY361 (*spt21Δ*), PKY329 (*spt21Δ hir1Δ*), PKY360 (*cac1Δ spt21Δ*), PKY302 (*cac1Δ hir1Δ*), and JRY4470 (*sir2Δ*) were used. Tenfold serial dilutions of each strain were spotted onto nonselective (YPAD) medium to observe the total number of cells and onto FOA medium to observe cells capable of telomeric silencing. For each independent experiment, the fraction of viable cells resistant to FOA (FOA^R) was normalized to the value obtained for the wild-type (wt) strain; the averages of data from three experiments ± standard deviations are shown. <, no FOA-resistant colonies were observed (of 2 × 10⁶ to 4 × 10⁶ plated). (B) Strains containing the *HMR*⁺::*ADE2* allele (68) were grown on YPD medium without additional adenine. A dark colony color indicates silencing of the *ADE2* gene. Strains PKY268 (wild type [wt]), PKY269 (*cac1Δ*), and PKY375 (*cac1Δ hir1Δ*) were used. (C) Strains PKY730 (*MATa hm1Δ sir1Δ cac2Δ hir3Δ*) (left) and PKY364 (*MATa HMLα sir1Δ cac2Δ hir3Δ*) (right) were replica plated onto YPD medium to test for growth (upper panel) or onto synthetic dextrose medium spread with a lawn of mating tester strain 217 (*MATα his1*) to select for diploid formation (lower panel).

TABLE 3. Mating efficiencies of strains with different combinations of *sir1Δ*, *cac2Δ*, and *hir3Δ* deletions

Strain genotype	Mating efficiency in background ^a :	
	<i>MATα</i>	<i>MATa</i>
Wild type	1.0 (3)	1.0 (5)
<i>cac2Δ</i>	1.2 ± 0.34 (3)	0.83 ± 0.34 (4)
<i>hir3Δ</i>	0.69 ± 0.35 (3)	0.91 ± 0.12 (4)
<i>sir1Δ</i>	0.29 ± 0.15 (3)	0.38 ± 0.13 (4)
<i>sir1Δ cac2Δ</i>	0.50 ± 0.18 (3)	3.3 × 10 ⁻² ± 2.0 × 10 ⁻² (4)
<i>sir1Δ hir3Δ</i>	0.54 ± 0.12 (3)	0.55 ± 0.18 (4)
<i>cac2Δ hir3Δ</i>	1.0 (2)	9.4 × 10 ⁻² ± 3.6 × 10 ⁻² (5)
<i>sir1Δ cac2Δ hir3Δ</i>	6.2 × 10 ⁻² ± 5.9 × 10 ⁻² (3)	1.9 × 10 ⁻⁴ ± 1.4 × 10 ⁻⁴ (5)

^a In each independent experiment, data were normalized to values obtained simultaneously for the wild-type strain. The values are averages of data from multiple experiments (numbers in parentheses) ± standard deviations. The data for the *MATα cac2Δ hir3Δ* strain are averages of values from two experiments, presented without standard deviations.

a complete loss of silencing at the *HM* loci (55). In contrast, in *sir1* mutant cells, *HML* silencing is mitotically metastable, with transcription of the resident genes switching between the repressed and active states, resulting in a population of genetically identical cells, each of which exists in one of two epigenetic states (51). This observation is consistent with the idea that the *SIR1* gene is required for efficient establishment of the silenced state at *HM* loci, which normally occurs in virtually every cell. *sir1* cells have a mild mating defect (55) (Table 3). However, several mutations which individually cause no mating deficiency cause synergistic reductions in silencing when combined with *sir1Δ* mutations (see, e.g., reference 54). Thus, *sir1Δ* mutants provide a sensitized background for the detection of silencing defects at the *HM* loci.

To test whether *cacΔ* or *hirΔ* mutations enhance the *sirΔ* mating defect, we quantitated the mating efficiencies of strains that contained all possible combinations of *SIR1*, *CAC2*, and *HIR3* gene deletions (Table 3). *MATa sir1Δ hir3Δ* mutants mated at the same levels as *sir1Δ* cells. In contrast, *MATa sir1Δ cac2Δ* cells mated approximately 10-fold less efficiently than *MATa sir1Δ* cells, indicative of a role for *CAC2* in *HM* silencing that is different from that of *SIR1*. This defect was not observed in *MATα sir1Δ cac2Δ* cells, presumably due to the stronger silencing at the *HMRa* locus. A dramatic loss of silencing was observed at *HMLα* in *MATa sir1Δ cac2Δ hir3Δ* triple mutants, which mated approximately 100-fold less efficiently than *MATa sir1Δ cac2Δ* double mutants. The *sir1Δ cac2Δ hir3Δ* combination also weakened silencing at *HMRa* sufficiently to reduce mating in a *MATα* strain approximately eightfold compared to *sir1Δ cac2Δ* mutants. To confirm that the reduction in mating efficiency observed in the *sir1Δ cac2Δ hir3Δ* triple mutants was due to a loss of silencing at *HM* loci and not to inactivation of the pheromone signaling pathway, we tested whether deletion of *HMLα* would restore mating. Indeed, deletion of *HML* restored mating to a *MATa sir1Δ cac2Δ hir3Δ* strain (Fig. 3C), indicating that coexpression of *a* and *α* genes is the cause of the mating defects in the triple mutant. Together, these data demonstrate that loss of both *CAC* and *HIR* genes synergistically weakens silencing at both telomeres and the *HM* loci and that the role of *CAC* and *HIR* genes does not overlap with that of *SIR1*.

Defects in telomeric chromatin structure. Position-dependent gene silencing at yeast telomeres and *HM* loci results in changes in chromatin structure as revealed by increased protection from nucleases and methylases (10, 36, 61). To test whether the reductions in telomeric gene silencing in *cacΔ hirΔ*

mutants are related to chromatin structure, we expressed the bacterial dam methylase gene in various yeast strains and tested the DNA of these strains for digestion by methylation-sensitive restriction enzymes (Fig. 4). The DNA was hybridized with a *URA3* DNA probe, which recognizes both the mutant *ura3-1* gene at the normal chromosomal position and the *URA3* gene inserted next to the chromosome VIII telomere. Figure 4A illustrates the expected sizes of DNA fragments from both loci; previous work has established that the GATC sequence internal to the telomeric copy of the *URA3* gene is largely protected from methylation in wild-type but not *sir2Δ* mutant cells, which lack telomeric silencing (10, 33). Methylase access at this sequence was assayed by digestion with the restriction enzyme *DpnI*, which requires methylation of the GATC recognition sequence for activity. Conversely, *DpnII* digestion of GATC sequences is inhibited by methylation. *Sau3AI* digestion of GATC sequences is insensitive to dam methylation and thus serves as a positive control for digestion.

A convenient metric for quantifying methylase accessibility at the telomere is the ratio of the band B signal to the total signal in the *HindIII*- and *DpnI*-digested samples (Fig. 4A, second lane in each set of four); complete resistance to digestion by the methylation-dependent enzyme *DpnI* at the GATC site within the telomeric *URA3* gene would result in a value of 0.5, because the endogenous *ura3-1* locus is known to be fully accessible to the methylase (33). We observed average ratios of 0.33 and 0.06 in wild-type and *sir2Δ* cells, respectively. In *cac1Δ* and *cac2Δ* cells, there was a decrease in this ratio compared to that of wild-type cells, to 0.22 and 0.21, respectively. In contrast, the ratio in *hir1Δ* cells was 0.40. Thus, the extent of methylase access in these strains has the same rank order as the strength of telomeric silencing. Consistent with this correlation, we observed a ratio of 0.12 for *cac2Δ hir1Δ* cells, well below the value observed for *cacΔ* cells but not as low as that for *sir2Δ* cells. We also digested DNA samples with the methylase-sensitive enzyme *DpnII* (third lane for each strain). Liberation of fragment C from the telomeric locus, reflecting protection from methylase activity, was greatly inhibited in *cac2Δ hir1Δ* and *sir2Δ* cells. We also noted that none of the mutants analyzed exhibited substantial changes in telomere length, as shown by the migration of the telomeric fragment labeled "T" in the *HindIII*-digested samples (first lane for each strain). Together, these data demonstrate that *cac2Δ hir1Δ* cells are defective in forming the telomeric chromatin structure required for gene silencing but are able to maintain wild-type telomere length.

Effects of changes in histone gene copy number on telomeric gene silencing. We have considered two classes of models to explain the synergistic loss of chromatin-mediated gene silencing in *cacΔ hirΔ* and *cacΔ spt21Δ* double mutants. First, the *HIR* genes and *SPT21* could encode chromosome assembly factors that act in a pathway partially redundant to CAF-I, so that severe silencing defects are not observed until both classes of genes are mutated. A second possibility is that *cacΔ* mutants are sensitive to the loss of these genes because nucleosome assembly pathways that operate in the absence of CAF-I are intrinsically sensitive to perturbations in histone levels. Both *hirΔ* and *spt21Δ* mutations cause misregulation of histone synthesis: three of the four histone gene loci are derepressed in *hirΔ* mutants (48, 65), while the histone *HTA2-HTB2* and *HHT2-HHF2* gene pairs are not fully activated in *spt21Δ* mutants (5). Thus, although *hirΔ* and *spt21Δ* mutations cause misregulation of histone gene transcription in opposite directions, both sets of mutations share the property of altering histone levels and,

FIG. 4. Increased accessibility of dam methylase to telomeric chromatin in a *cac2Δ hir1Δ* double mutant. (A) Diagram of the *URA3* gene adjacent to the chromosome VIII telomere [(TG)_n] and the *ura3-1* gene at the natural locus on chromosome V (33, 78). Restriction sites for *Hind*III and 5'-GATC-3' sequences are indicated by H3 and Sau, respectively. The GATC sequence that is more protected from methylation in wild-type cells than in *sir* cells is indicated by Sau*. Expected restriction fragments (A, B, C, and the telomeric fragment T) are shown schematically. (B) Strains expressing bacterial dam methylase were PKY300 (*CAC*⁺), PKY299 (*cac1Δ*), PKY305 (*cac2Δ*), PKY310 (*cac2Δ hir1Δ*), PKY311 (*hir1Δ*), and AJL387-5a (*sir2Δ*). DNA from each strain was digested with four different combinations of restriction enzymes prior to agarose gel electrophoresis and DNA hybridization with a *URA3* probe. Shown from left to right, for each strain: digestion with *Hind*III alone, digestion with *Hind*III plus *Dpn*I, digestion with *Hind*III plus *Dpn*II, digestion with *Hind*III plus *Sau*3AI. "DpnI protection" indicates the fraction of total counts in the *Hind*III plus *Dpn*I lanes present in the B restriction fragment, indicating protection of the GATC site internal to the telomeric *URA3* gene from dam methylase. The values presented are averages of data from two independent experiments.

in some instances, histone stoichiometry (59). Note that the two models proposed are not mutually exclusive.

To test for effects of histone levels on the TPE in *cacΔ* mutants, we performed two types of experiments. We first overexpressed individual histone gene pairs (*HTA-HTB* or *HHT-HHF*) in wild-type and *cac2Δ* cells and measured the TPE in the transformants (Table 4). Northern blots showed that overexpressed histone mRNAs accumulated to similar levels in wild-type and *cac2Δ* cells (data not shown), again indicating that CAF-I does not regulate histone gene expression. In wild-type cells, overexpression of any of the four histone gene pairs resulted in TPE levels equivalent to those of control strains carrying the vectors alone. As previously observed, *cac2Δ* mutants displayed reduced TPE levels (28) (Fig. 5A). Unlike the case for wild-type cells, telomeric silencing in *cac2Δ* mutants was either increased or further reduced depending on the types of histones that were overexpressed. Overexpression of histones H2A and H2B resulted on average in a threefold (*HTA2-HTB2*) or an eightfold (*HTA1-HTB1*) reduction in telomeric

silencing. In contrast, overexpression of histones H3 and H4 from either *HHT-HHF* gene pair resulted in a threefold stimulation of silencing. Furthermore, overexpression of all four core histones (using the *HHT1-HHF1* and *HTA1-HTB1* gene pairs) simultaneously led to a greater than fourfold enhancement of silencing in *cac2Δ* mutants, approaching the level of silencing in a wild-type strain in these experiments. These data suggest that TPE is more sensitive to changes in histone levels in the absence of CAF-I. In addition, the opposing effects of H2A-H2B versus H3-H4 overexpression on the TPE in *cac2Δ* mutants further suggest that CAF-I-independent pathways are selectively perturbed by an altered histone stoichiometry.

We also tested the effects on TPE of a reduction in the histone gene copy number. Deletion of either gene pair encoding histones H3 and H4 or deletion of only the *HHF2* gene, which encodes histone H4, did not reduce silencing in an otherwise wild-type strain (Fig. 5A). In contrast, any of these histone H3-H4 deletions caused a strong synergistic loss of telomeric silencing if combined with a *cac2Δ* deletion [approx-

TABLE 4. Effects of histone overexpression on telomeric silencing in wild-type and *cac2Δ* cells

Strain	Relevant genotype ^a	FOA/total cell ratio ^b	n
PKY367	wt + YEp351	0.51 ± 0.091	6
PKY368	wt + YEp351-(HTA1-HTB1)	0.55 ± 0.14	3
PKY451	wt + YEp351-(HHT1-HHF1)	0.54 ± 0.11	3
PKY460	wt + YEp351-(HHT1-HHF1 + HTA1-HTB1)	0.72 ± 0.44	3
PKY454	wt + YEplac181	0.66 ± 0.22	3
PKY452	wt + YEplac181-(HHT2-HHF2)	0.59 ± 0.22	3
PKY453	wt + YEplac181-(HTA2-HTB2)	0.55 ± 0.095	3
PKY369	<i>cac2Δ</i> + YEp351	0.10 ± 0.047	6
PKY370	<i>cac2Δ</i> + YEp351-(HTA1-HTB1)	0.013 ± 0.0095	3
PKY455	<i>cac2Δ</i> + YEp351-(HHT1-HHF1)	0.31 ± 0.040	3
PKY461	<i>cac2Δ</i> + YEp351-(HHT1-HHF1 + HTA1-HTB1)	0.46 ± 0.082	3
PKY458	<i>cac2Δ</i> + YEplac181	0.066 ± 0.012	3
PKY456	<i>cac2Δ</i> + YEplac181-(HHT2-HHF2)	0.20 ± 0.053	3
PKY457	<i>cac2Δ</i> + YEplac181-(HTA2-HTB2)	0.021 ± 0.011	3

^a wt, wild type.^b Telomeric silencing of the *URA3-VIIL* reporter was assayed as described in the legend to Fig. 3, except that the media used were synthetic complete medium (SC) minus leucine and SC minus leucine plus FOA in order to maintain selection for the plasmids. For each strain, the fraction of Leu⁺ cells resistant to FOA was measured; the averages of data from *n* experiments ± standard deviations are shown.

imately 100-fold in the case of (*hht1-hhf1*)Δ *cac2Δ* compared to *cac2Δ*; other combinations resulted in larger effects (Fig. 5A), similar to the multiple-order-of-magnitude effects observed in *cacΔ hirΔ* mutants (Fig. 3). Although silencing was reduced to similar extents in *cacΔ* strains on deletion of either *HIR* or H3-H4 genes, no 37°C growth defects were associated with H3-H4 deletions, even when combined with *cacΔ* deletions (data not shown). We also determined the effects of deleting the gene pairs encoding histones H2A and H2B. However, deletion of the *HTA1-HTB1* gene pair caused lethality in our strain background (data not shown), even though the same deletion allele has been used to construct viable haploid strains in other backgrounds (17). Deletion of the other gene pair, *HTA2-HTB2*, was not lethal and did not have a statistically significant effect on telomeric silencing in an otherwise wild-type cell (Fig. 5B). Telomeric silencing in an (*hta2-htb2*)Δ *cac1Δ* double mutant was slightly reduced, on average, relative to that of a *cac1Δ* mutant, although the standard deviations in these experiments were overlapping. However, this may be an underestimate of the silencing defect because of dosage compensation by the remaining *HTA1-HTB1* gene pair (43). Together, these data suggest that telomeric silencing is most significantly perturbed by reduction of the level of histone H3-H4 expression in the absence, but not in the presence, of CAF-I.

Telomeric silencing is largely unaffected by misregulation of *HTA1-HTB1* transcription. The previous data do not rule out the possibility that the Hir proteins also directly function with CAF-I in formation of heterochromatin. To test this possibility, we constructed strains in which the *cis*-acting negative site within the *HTA1-HTB1* promoter was deleted (*HTA1Δ*-neg mutants). The Hir proteins negatively regulate expression of this gene pair through this site (48); the resulting cells thus constitutively express the *HTA1* and *HTB1* genes, yet they contain functional Hir proteins. We measured the TPE in these strains in the presence and absence of the *CAC1* gene to determine whether misregulation of the *HTA1* and *HTB1* genes in the presence of functional Hir proteins was sufficient to cause a synergistic loss of TPE in a *cac1Δ* mutant. We observed that the *HTA1Δ*-neg mutation had no effect on TPE itself (Fig. 5C), consistent with the observation that *hirΔ* mutants, which cannot negatively regulate *HTA1* synthesis, also do not have TPE defects (Fig. 3). The *HTA1Δ*-neg *cac1Δ* double mutant displayed a TPE level approximately twofold lower than that of a *cac1Δ* strain, a decrease similar to that observed

in a *cacΔ* mutant on overproduction of histones H2A and H2B (Table 4). However, the TPE in the *HTA1Δ*-neg *cac1Δ* double mutant was much stronger than that observed in *cacΔ hirΔ* strains (Fig. 3). We conclude that misregulation of the *HTA1-HTB1* gene pair does not contribute in a major way to the synergistic loss of telomeric silencing observed in *cacΔ hirΔ* strains.

DISCUSSION

Multiple contributions to heterochromatin formation. Yeast cells lacking CAF-I have reduced levels of telomeric gene silencing (9, 28). In this study, we found that *cacΔ* mutants also have subtle defects in silencing at the silent mating type loci *HMR* and *HML* that can be detected with sensitive assays (Fig. 3C; Table 3), consistent with other recent studies (8). The extensive residual silencing observed in *cacΔ* mutants suggests that CAF-I is partially redundant to other factors which participate in heterochromatin formation. The defect in silencing in *cacΔ* mutants appears to be related to the stability of the silenced chromatin once it is formed, because *cacΔ* mutants activate previously repressed telomere-proximal genes at a higher frequency (42) and more rapidly escape cell cycle arrest caused by exposure to alpha mating pheromone, a sensitive assay for the stability of *HML* silencing (8). These findings are consistent with the small colony size of FOA-resistant *cacΔ* mutants in TPE assays (Fig. 3) (9, 28) and the variegated appearance of *cacΔ HMR::ADE2* strains on adenine-limited media (Fig. 3B). The synergistic reduction of *HML* silencing in *sir1Δ cacΔ* double mutants (Table 3) (8) thus may result from a loss of factors that contribute to two aspects of silencing: establishment of the silenced state (Sir1p [51]) and maintenance of heterochromatin (CAF-I). However, because the defect in *HML* silencing in *sir1Δ cacΔ* double mutants is partial, there must be other factors that contribute to silencing in the absence of Sir1p and CAF-I.

Several lines of evidence presented here suggest that the Hir proteins represent one set of factors that cooperate with CAF-I in the formation of heterochromatin. Combination of *hirΔ* and *cacΔ* gene deletions caused dramatic and synergistic reductions in silencing at both telomeres and *HM* loci (Fig. 3; Table 3). Moreover, these phenotypes correlated with changes in chromatin structure that have been associated with a loss of silencing (10, 33, 61): the telomeric chromatin of a *cac2Δ hirΔ*

FIG. 5. Changes in histone gene dosage and expression affect telomeric gene silencing in a *cacΔ* mutant but not in wild-type cells. Telomeric silencing of the *URA3-VIII* reporter was assayed as described in the legend to Fig. 3. For each strain, the fraction of viable cells resistant to FOA (FOA^R) was normalized to the value obtained for the wild-type strain; the averages of values from *n* experiments $\pm$ the standard deviations are shown, except for the *hhf2Δ cac2Δ* strain, for which the averages of data from two experiments are presented. (A) Deletion of histone H3 and H4 genes. Strains PKY090 (wild type [wt]), PKY107 (*cac2Δ*), PKY408 [(*hht1-hhf1*) Δ], PKY409 [(*hht1-hhf1*) Δ *cac2Δ*], PKY410 [(*hht2-hhf2*) Δ], PKY411 [(*hht2-hhf2*) Δ *cac2Δ*], PKY412 (*hhf2Δ*), and PKY413 (*hhf2Δ cac2Δ*) were used. (B) Deletion of the *HTA2-HTB2* gene pair encoding histones H2A and H2B. Strains PKY090 (wt), PKY106 (*cac1Δ*), PKY499 [(*hta2-htb2*) Δ], and PKY500 [(*hta2-htb2*) Δ *cac1Δ*] were used. (C) Deletion of the negative regulatory site in the *HTA1-HTB1* promoter. Strains PKY090 (wt), PKY106 (*cac1Δ*), PKY581 (*HTA1Δneg*), and PKY582 (*HTA1Δneg cac1Δ*) were used.

mutant displayed a large increase in accessibility to dam methylase relative to that of mutants lacking either gene alone (Fig. 4).

Disruption of *HIR* genes alone had little, if any, effect on gene silencing at telomeres or the silent *HM* loci (Fig. 3; Table

3). One interpretation of these data is that the role of Hir proteins in gene silencing is functionally redundant to other mechanisms. However, in the absence of Hir proteins, histone synthesis is misregulated, resulting in the constitutive transcription of both *HHT-HHF* loci and one *HTA-HTB* locus (48,

60, 65). It is therefore possible that *hir* Δ mutants do have defects in heterochromatin function but that these defects are phenotypically masked by the simultaneous overexpression of the four core histone proteins, such as we observed in a *cac2* Δ strain transformed with high-copy-number *HTA-HTB* plus *HHT-HHF* genes (Table 4).

Role of *HIR* genes in silencing through regulation of histone synthesis. Why is silencing in *cac* Δ mutants sensitive to the loss of the *HIR* genes? One possible explanation is that Hir proteins and CAF-I play partially redundant roles in the formation of heterochromatin and that the loss of both factors is required to observe dramatic decreases in silencing. Because *hir* Δ mutants misregulate expression of a subset of the yeast histone genes (48, 60), a second hypothesis proposes that changing the absolute levels or the relative stoichiometry of the core histones would be detrimental to gene silencing in the absence of CAF-I.

Several lines of evidence suggest that the second hypothesis is correct. Telomeric silencing in *cac* Δ mutants, but not in wild-type cells, is strongly reduced by deletion of genes encoding histones H3 and H4 (Fig. 5A) and is moderately reduced by *H2A-H2B* overexpression (Table 4). In contrast, deletion of the *HTA2-HTB2* gene pair encoding H2A-H2B had a slight effect on silencing in a *cac* Δ mutant (Fig. 5B), and the TPE in a *cac2* Δ strain was enhanced by overproduction of histones H3 and H4 (Table 4). Therefore, we also propose that deletion of *SPT21*, which is required for expression of both *HTA2-HTB2* and *HHT2-HHF2* (5), causes a synergistic loss of telomeric silencing in *cac* Δ mutants because of the loss of expression of the *HHT2-HHF2* gene pair (Fig. 3).

Together, these data demonstrate that silencing in *cac* Δ mutants is sensitive to both the overall level and the relative stoichiometry of histone proteins. We suggest that this reflects the sensitivity of an alternative silencing pathway(s) to altered histone levels. When the *CAC* genes are intact, this alternative pathway is not required for telomeric or *HM* silencing, so changes in histone gene expression have little effect on the formation of heterochromatin. However, in *cac* Δ mutants, this pathway plays a substantial role in silencing, and it is easily perturbed by changes in histone levels or stoichiometry. Because human CAF-I is known to be a histone H3-H4 binding and deposition factor (63, 74, 75), we expect that the redundant yeast pathway also performs this function. When cellular levels of histones H3 and H4 fall below a certain threshold, as might occur in the case of an *HHT-HHF* or *SPT21* gene deletion, this pathway appears to function inefficiently, analogous to the functioning of an enzyme when the substrate concentration is far below the K_m value. Note that overexpression of histones H3 and H4 in a *cac* Δ mutant slightly improved the TPE, consistent with an improved function of a putative backup pathway (Table 4). In contrast, overexpression of histones H2A and H2B in a *cac* Δ mutant further reduced telomeric silencing (Table 4). We propose that this occurs by sequestration of unassembled (H3-H4)₂ tetramers, because this phenotype is suppressed by co-overexpression of H3-H4. Changes in histone stoichiometry have also been shown to reduce mitotic chromosome stability (40). In that case, overproduction of either H2A-H2B or H3-H4 led to increased chromosome loss but simultaneous overproduction of all four core histone proteins did not. This suggests that histone stoichiometry, but not necessarily the absolute levels of histones, is important for preventing chromosome loss. In contrast, in the absence of CAF-I, heterochromatin formation is sensitive both to the levels of H3-H4 and to the stoichiometry of the four core histones. CAF-I ensures that heterochromatin formation oc-

curs efficiently even in cells that exhibit abnormalities in histone gene expression.

Our data are also consistent with the possibility that the CAF-I-independent silencing pathway is not mediated by assembly proteins and that histones are able to assemble properly on DNA on their own provided that they are present in a sufficiently high concentration. Although we cannot presently rule out this possibility, we do not favor it because studies of several organisms have found that unassembled histone proteins are complexed with other proteins, including factors involved in the deposition of histones onto DNA (2, 4, 20, 31, 75).

Global defects in *cac* Δ *hir* Δ mutants. In addition to causing reductions in heterochromatic gene silencing, simultaneous deletion of the *CAC* and *HIR* genes revealed other phenotypes that suggest more-global chromosomal roles for CAF-I and the Hir proteins. *cac* Δ *hir* Δ mutants displayed growth defects at 30°C that were exacerbated at both high (37°C [Fig. 2; Table 2]) and low (16°C [data not shown]) temperatures. The histone H3-H4 gene pair deletions did not cause synergistic growth defects when combined with *cac* Δ mutations (data not shown), suggesting that the growth defects in *cac* Δ *hir* Δ mutants neither caused nor arose from a loss of heterochromatic silencing. Furthermore, deletion of both the *HIR1* and *HIR2* genes in *cac* Δ mutants increased the generation time at 30°C and reduced the viability at 37°C (Table 2). Because histone genes are no more derepressed in *hir1* Δ *hir2* Δ double mutants than in the corresponding single mutants (58), this suggests that the synthetic growth phenotypes do not solely result from changes in histone gene expression, although subtle effects on histone levels may not be detected.

Formation of heterochromatin is not required for yeast viability; *sir2*, *sir3*, and *sir4* mutants completely lack telomeric and *HM* silencing (1, 55) and have wild-type growth rates (54a; see also reference 46). Therefore, the growth defects observed in *cac* Δ *hir* Δ mutants suggest the existence of a more global defect in chromosomes. It remains to be determined whether these growth phenotypes result from changes in gene expression, structural defects in chromosomes, or both. Global defects are also consistent with data showing that a combination of *cac* Δ and *hir* Δ mutations results in a synergistic stimulation of the rate of Ty transposition (52).

Do Hir proteins directly contribute to (hetero)chromatin formation? It is unlikely that *cac* Δ and *hir* Δ mutations act synergistically to cause repression of genes required for silencing (e.g., *SIR* genes or assembly factor genes), because there exists no evidence that CAF-I or Hir proteins act as transcriptional activators; indeed, when tethered to heterologous promoters, Hir proteins are general repressors of transcription (65). Furthermore, *cac* Δ mutants display no phenotypes common to other mutants lacking transcriptional activation functions, such as failure to grow on alternative carbon sources or amino acid auxotrophies, nor do they have an Spt⁻ phenotype (data not shown). Thus, the synergistic effect of *cac* Δ and *hir* Δ mutations on gene silencing is likely to result from a shared defect in chromosome function.

What overlapping role in chromosome function might be shared by CAF-I and Hir proteins? One possibility is that Hir proteins and CAF-I are both histone deposition factors. The Hir proteins are sensitive monitors of the intracellular levels of histones H2A and H2B and modulate *HTA1-HTB1* transcription in response to altered levels of these histones (43). This suggests that they may be histone binding proteins. Indeed, Hir2p can bind directly to immobilized H3, H4, and H2B molecules (53a), suggesting that repression of the *HTA1-HTB1* promoter could be mediated through histone deposition by Hir

protein complexes (3a). We note that heterochromatic regions in *S. cerevisiae* contain all four core histones (15) and that human CAF-I directly binds and deposits histones H3 and H4 (63, 75). A simple hypothesis would be that Hir proteins are among several factors able to deposit histones H2A and H2B to complete nucleosome formation initiated by CAF-I or other activities. An alternative hypothesis is that Hir proteins are structural components of chromatin and that their presence in heterochromatin marks it for nucleosome assembly by another histone deposition factor when CAF-I is absent. In either model, the CAF-I and Hir protein complexes might function in partially overlapping ways to form repressive nucleosomal structures at some chromosomal locations, such that the loss of both complexes results in synergistic defects in chromatin formation.

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