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Ultraviolet radiation sensitivity and reduction of telomeric silencing in *Saccharomyces cerevisiae* cells lacking chromatin assembly factor-I

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In vivo, nucleosomes are formed rapidly on newly synthesized DNA after polymerase passage. Previously, a protein complex from human cells, termed chromatin assembly factor-I (CAF-I), was isolated that assembles nucleosomes preferentially onto SV40 DNA templates that undergo replication *in vitro*. Using a similar assay, we now report the purification of CAF-I from the budding yeast *Saccharomyces cerevisiae*. Amino acid sequence data from purified yeast CAF-I led to identification of the genes encoding each subunit in the yeast genome data base. The *CAC1* and *CAC2* (chromatin assembly complex) genes encode proteins similar to the p150 and p60 subunits of human CAF-I, respectively. The gene encoding the p50 subunit of yeast CAF-I (*CAC3*) is similar to the human p48 CAF-I subunit and was identified previously as *MSI1*, a member of a highly conserved subfamily of WD repeat proteins implicated in histone function in several organisms. Thus, CAF-I has been conserved functionally and structurally from yeast to human cells. Genes encoding the CAF-I subunits (collectively referred to as *CAC* genes) are not essential for cell viability. However, deletion of any *CAC* gene causes an increase in sensitivity to ultraviolet radiation, without significantly increasing sensitivity to gamma rays. This is consistent with previous biochemical data demonstrating the ability of CAF-I to assemble nucleosomes on templates undergoing nucleotide excision repair. Deletion of *CAC* genes also strongly reduces silencing of genes adjacent to telomeric DNA; the *CAC1* gene is identical to *RLF2* (Rap1p localization factor-2), a gene required for the normal distribution of the telomere-binding Rap1p protein within the nucleus. Together, these data suggest that CAF-I plays a role in generating chromatin structures *in vivo*.

[Key Words: Nucleosome; DNA damage; heterochromatin; DNA replication]

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The first chromatin proteins deposited onto nascent DNA are the core histones, resulting in formation of nucleosomes (Worcel et al. 1978). Nucleosomes are the fundamental repeat structures of chromatin and are comprised of 146 bp of DNA wrapped around an octamer of histone proteins (two molecules of each of the core histones H2A, H2B, H3, and H4; for review, see van Holde 1989). In the budding yeast *Saccharomyces cerevisiae*, DNA replication in the absence of expression of one of the core histones results in a loss of viability that cannot be rescued by later expression of the missing histone (Han et al. 1987; Kim et al. 1988). Therefore, proper coordination between DNA replication and nucleosome assembly is an essential process.

During DNA replication, existing nucleosomes on the parental DNA are segregated randomly onto the two nascent DNA duplexes (Sogo et al. 1986; for review, see Kaufman and Botchan 1994; Krude 1995b). During each round of DNA replication, half of the nucleosomes are formed from newly synthesized histones in two steps. Newly synthesized histones H3 and H4 are deposited onto DNA first, followed by histones H2A/H2B, to complete the nucleosome (Worcel et al. 1978; Jackson 1990; Smith and Stillman 1991). Existing nucleosomes on the parental DNA template are segregated to nascent DNA efficiently *in vitro* in highly purified DNA replication systems (Ishimi et al. 1991). In contrast, additional cellular factors are required for deposition of newly synthesized histones during DNA replication (for review, see Kaufman and Botchan 1994; Krude 1995b).

It is presently unknown which proteins mediate assembly of histones onto DNA *in vivo*. Biochemical ap-

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proaches to this problem have often used extracts of egg or embryonic cells from *Xenopus* and *Drosophila* (Laskey et al. 1977; Glikin et al. 1984; Becker and Wu 1992; Kamakaka et al. 1993). Such extracts assemble nucleosomes efficiently because embryos typically contain a large maternal store of histones bound to acidic "chaperone" proteins. Several of the proteins from *Xenopus* that mediate nucleosome assembly in vitro have been purified and cloned (Kleinschmidt et al. 1986; Dingwall et al. 1987); however, these factors do not require DNA synthesis to assemble nucleosomes. Therefore, these factors operate by mechanisms that do not reflect adequately the observed link between replication fork passage and assembly of nucleosomes in mammalian somatic cells. Also, in contrast to embryonic cells, somatic cells generally do not store large pools of histones. Instead, most histones are synthesized in a cell cycle-dependent manner at the onset on DNA synthesis, just before de novo nucleosome assembly occurs (Wu and Bonner 1981; Russev and Hancock 1982; Jackson 1990). Therefore, an understanding of how chromatin assembly is regulated in somatic cells requires functional characterization of proteins that link assembly of nucleosomes to DNA replication.

A three-subunit protein complex termed chromatin assembly factor-I (CAF-I) was purified from human somatic cell nuclear extracts based on its ability to assemble histone octamers onto SV40 origin-based plasmids undergoing replication in vitro (Stillman 1986; Smith and Stillman 1989; for review, see Krude 1995b; Kaufman 1996). CAF-I differs from other assembly factors because it assembles nucleosomes preferentially on DNA that has recently undergone DNA replication (Smith and Stillman 1989; Kaufman et al. 1995; Kamakaka et al. 1996). CAF-I performs the first step of the assembly process, bringing histones H3 and H4 to replicating DNA; histones H2A/H2B can bind to this "chromatin precursor" subsequent to DNA replication to complete the histone octamer (Smith and Stillman 1991). Thus, CAF-I assembles nucleosomes in vitro by the same two-step mechanism observed in vivo, and it does so in a manner linked to DNA replication. The nature of the molecular signal that governs DNA replication-preferential activity of CAF-I is not known presently. CAF-I forms nucleosomes for a short period after inhibition of DNA synthesis (Kamakaka et al. 1996), and can perform nucleosome assembly on DNA templates undergoing nucleotide excision repair (Gaillard et al. 1996). These data are consistent with a model in which CAF-I is activated by proteins or nucleic acid structures, or both, left in the wake of DNA polymerases (Kaufman 1996). In S-phase nuclei of human cells, CAF-I colocalizes with punctate DNA replication foci (Krude 1995a), suggesting that CAF-I plays a role in the new nucleosome assembly at least at some of the clustered DNA replication forks at each focus.

Human CAF-I is comprised of subunits termed p150, p60, and p48 (Smith and Stillman 1989; Verreault et al. 1996). p150 is an acidic protein with several highly charged regions; the other subunits, p60 and p48, contain

WD repeats (Kaufman et al. 1995; Verreault et al. 1996), a prevalent repeating motif in many eukaryotic proteins (Neer et al. 1994). The p48 subunit is a member of a highly conserved subfamily of WD proteins. This subfamily has been implicated in multiple aspects of histone metabolism (Parthun et al. 1996; Taunton et al. 1996; Verreault et al. 1996). In this paper we have purified a DNA replication-linked chromatin assembly factor from *S. cerevisiae* to study formation of chromosomes by both biochemical and genetic techniques. This approach led to the identification of a yeast CAF-I protein complex that is molecularly and structurally similar to that purified from human cells.

Results

Detection and purification of a yeast DNA replication-linked chromatin assembly factor

Although candidate homologs of the human CAF-I subunits were present in yeast genome data base entries, the presence of multiple p48-related proteins in yeast made it impossible to identify genes involved in nucleosome assembly unambiguously. Therefore, we searched for biochemical activities from yeast cell extracts that assemble nucleosomes in a DNA replication-preferential manner during SV40 DNA replication in vitro. In this assay, DNA templates that undergo replication are labeled by incorporation of radioactive dATP. After DNA synthesis, proteins are removed from the DNA templates by phenol extraction, and the purified DNA is analyzed by agarose gel electrophoresis. Formation of nucleosomes during the synthesis stage is detected by an increase in the negative supercoiling of the purified DNA (see Stillman 1986 and references therein).

Previously, our biochemical interspecies complementation approach has allowed detection of CAF-I like-activities from *Drosophila* and *Xenopus* embryo extracts (Gaillard et al. 1996; Kamakaka et al. 1994, 1996). On the basis of the successful purification of other yeast proteins, whole cell extracts were prepared by glass bead-beating of late log-phase cells (Bell et al. 1993). Initial experiments showed that material from the initial cation exchange column that eluted at a conductivity equivalent to the starting buffer plus 0.27 M NaCl contained a replication-preferential nucleosome assembly activity that would be revealed after subsequent anion exchange chromatography (see Materials and Methods). This activity could not be detected reliably earlier in the purification because of replication-independent DNA supercoiling activities in chromatographic fractions from the first column, including the ABFII protein (data not shown; see Diffley and Stillman 1991).

As shown previously for human CAF-I (Smith and Stillman 1989), the yeast nucleosome assembly factor binds to columns of histone-agarose (Fig. 1A), although it is eluted at lower salt concentrations than human CAF-I. These chromatographic fractions were assayed. Comparison of the autoradiograph of synthetically labeled replication products with total DNA detected by ethid-

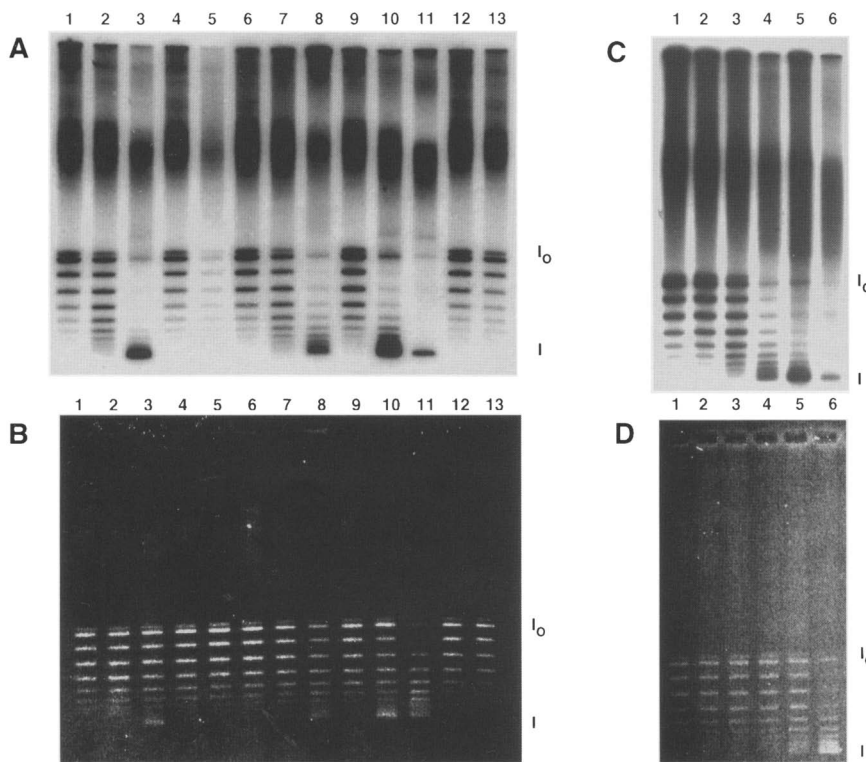


Figure 1. Identification of a histone-binding, DNA replication-preferential nucleosome assembly factor in yeast extracts. (A) Nucleosome assembly assay of histone-agarose chromatography fractions. A single-stranded DNA (ssDNA) cellulose column fraction was loaded onto a histone-agarose column and bound proteins were eluted with increasing NaCl as described in Materials and Methods. SV40 DNA replication reactions were performed with the addition of no additional protein (lane 1), input ssDNA fraction (0.5 and 2 μ l; lanes 2,3), histone-agarose column flowthrough (1 and 4 μ l; lanes 4,5), histone-agarose 0.2 M NaCl elution (0.04, 0.2, and 0.5 μ l; lanes 6–8), histone-agarose 0.4 M NaCl elution (0.04, 0.2, and 0.5 μ l; lanes 9–11), and histone-agarose 0.6 M NaCl elution (0.2 and 0.5 μ l; lanes 12,13). (I₀) The migration of topologically relaxed circular DNA templates; (I₁) The migration of supercoiled DNA indicative of nucleosome assembly. (B) Ethidium bromide stain of the gel shown in A. Even when sufficient CAF-I had been added to convert the majority of replicated DNA into a highly supercoiled species (A, lanes 3,8,10,11), not all of the unreplicated DNA molecules detected by

ethidium staining are supercoiled. This indicates preferential assembly of nucleosomes on the replicated DNA templates. (C) Titration of purified yeast CAF-I. Glycerol gradient-purified yeast CAF-I (fraction 5 from the gradient shown in Fig. 2) was titrated in the nucleosome assembly assay. The equivalents of 0, 0.15, 0.3, 0.6, 1.2, and 2.4 μ l of fraction 5 were added to 25- μ l replication reactions in lanes 1 through 6, respectively. (D) Ethidium bromide stain of the gel shown in C.

ium bromide staining (Fig. 1B) shows that replicated DNA was assembled preferentially into chromatin, as revealed by the formation of negatively supercoiled DNA (form I in Fig. 1 and 2). Human CAF-I displays a similar preference (Smith and Stillman 1989; Kaufman et al. 1995). The yeast assembly factor was purified extensively on a preparative scale by four chromatographic steps and glycerol gradient sedimentation (see Materials and Methods). When added at oversaturated amounts, a highly purified yeast assembly factor inhibits DNA synthesis and causes bulk supercoiling of input DNA (Fig. 1C,D, lane 6). This property is shared by the human and *Drosophila* CAF-I proteins (Kamakaka et al. 1996). However, at subsaturating amounts of yeast CAF-I (Fig. 1C,D, lanes 4,5), replicated molecules are supercoiled preferentially compared to the total input DNA.

To demonstrate that the supercoiling of DNA molecules by yeast assembly factor reflects assembly of nucleosomes, DNA replication products were analyzed by micrococcal nuclease (MNase) digestion. Limited digestion with MNase results in preferential cleavage of the linker DNA between nucleosomes; nucleosome assembly by human and *Drosophila* CAF-I results in protection of a ladder of DNA bands from MNase digestion (Smith and Stillman 1989; Kaufman et al. 1995; Kamakaka et al. 1996). We show here that recombinant human CAF-I and glycerol gradient-purified yeast assem-

bly factor generate replication products that display an identical pattern of MNase-resistant DNA (Fig. 2D). Therefore, we refer to the purified protein complex as yeast CAF-I.

Glycerol-gradient purified yeast CAF-I from several preparations consistently contained three polypeptides (p90, p60, and p50) that cosedimented in the glycerol gradient (Fig. 2A–C). Other proteins in similar gradient-purified preparations did not always peak in the same fraction as CAF-I activity (data not shown). As noted above, the high concentration of CAF-I in the peak fractions inhibited DNA replication (Fig. 2B, lanes 5–7). Sufficient amounts of each of the three candidate proteins were purified for amino acid sequence determination. Screening of the yeast genome data base with the peptide sequence data resulted in identification of the genes encoding each of the yeast CAF-I subunits. Each of these yeast genes is similar to the respective human CAF-I subunit gene (Fig. 3; also see Verreault et al. 1996). The genes encoding the p90, p60, and p50 subunits of yeast CAF-I were called *CAC1*, *CAC2*, and *CAC3*, respectively, because they were isolated as members of a chromatin assembly complex (the acronym *CAF* had been used previously for unrelated yeast genes). *CAC3* had been isolated previously and termed *MS11* (see below). All the peptides sequenced were found in these three genes.

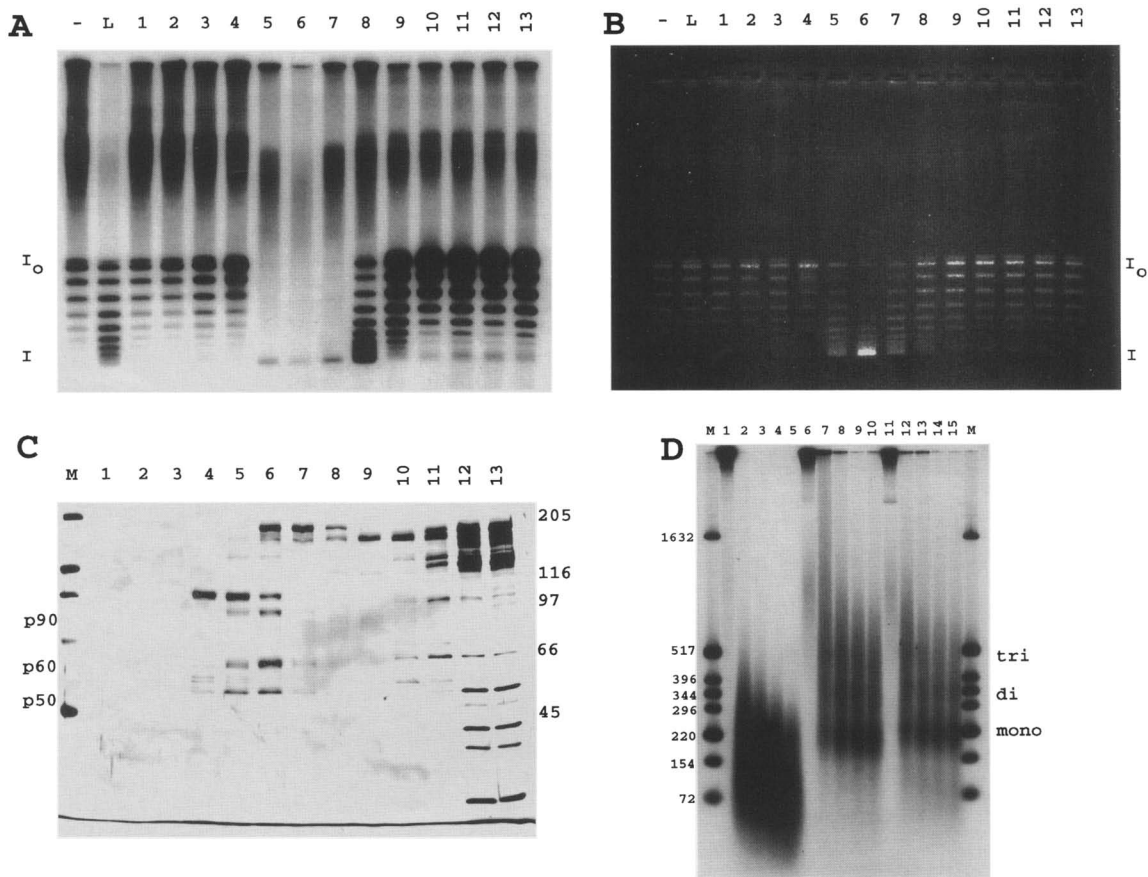


Figure 2. Preparative purification of yeast CAF-I. (A) Nucleosome assembly assay of glycerol gradient purified yeast CAF-I. SV40 DNA replication reactions were performed with the addition of glycerol gradient-purified yeast CAF-I (numbered lanes, corresponding to numbered protein fractions shown in C), material loaded onto the gradient (lane L), or no additional protein (lane -). DNA products were analyzed by agarose gel electrophoresis; an autoradiograph of synthetically labeled DNA is shown. Migration of topologically relaxed DNA (I_o) and negatively supercoiled DNA (I) is indicated; the material migrating slightly more slowly than the form I band in lanes 10–13 is unwound, and not supercoiled molecules. (B) Ethidium bromide stain of the gel shown in A. Note that when CAF-I was present but not oversaturated (e.g., lanes L, 5, 7), labeled monomeric DNA was supercoiled but not all of the DNA in the reaction was. This indicates preferential assembly of nucleosomes on the replicated DNA templates. (C) Proteins were analyzed by SDS-PAGE and stained with silver. Numbered lanes indicate gradient fractions, increasing from *top* to *bottom*. Lane M shows marker proteins (205, 116, 97, 66, and 45 kD from *top* to *bottom*, migration indicated to the *right*). The migration of the CAF-I p90, p60, and p50 subunits (peaking in fraction 6) is shown to the *left*. This preparation of protein was used for amino acid sequence analysis. (D) MNase digestion of replication products. SV40 DNA replication reactions (120 μ l) were supplemented with histones H2A and H2B (see Materials and Methods) and no assembly factor (lanes 1–5), recombinant human CAF-I p150 (60 ng; lanes 6–10) or 7 μ l of glycerol gradient-purified yeast CAF-I (fraction 5 above; lanes 11–15). Reaction products were digested with MNase for 0 min (lanes 1, 6, 11), 10 min (lanes 2, 7, 12), 20 min (lanes 3, 8, 13), 30 min (lanes 4, 9, 14), or 40 min (lanes 5, 10, 15) before deproteinization and electrophoresis through a 2% agarose gel. Molecular mass markers (M) with sizes in base pairs are indicated. Migration of mono-, di-, and trinucleosome length DNA is indicated.

Genes encoding yeast CAF-I subunits

The *CAC1* [Chromosome XVI open reading frame (ORF) YPR018w in the Yeast Protein Database] has also been isolated in a screen for genes affecting telomere function (see Discussion; Enomoto et al., this issue). Of the three CAF-I subunits, the yeast p90 protein is the least similar to its human counterpart, with ~25% identity (Fig. 3B). Like human p150, yeast p90 does contain highly charged lysine/glutamate/arginine-rich (KER) and glutamate/aspartate-rich (ED) regions. However, the proline/gluta-

mate/serine/threonine-rich (PEST) region found in human p150 is absent (Fig. 3A,B).

Like the human p60 protein, the yeast p60 (Cac2p) subunit contains WD-repeat sequences. However, it lacks the PEST region found at the carboxyl terminus of the human protein. In the WD-repeat region, the human and yeast proteins are 34% identical; inclusion of conservative changes increases the homology to 47%. More significantly, the alignment of the two proteins shows that the homology extends over the length of the entire WD-repeat region and includes residues other than those

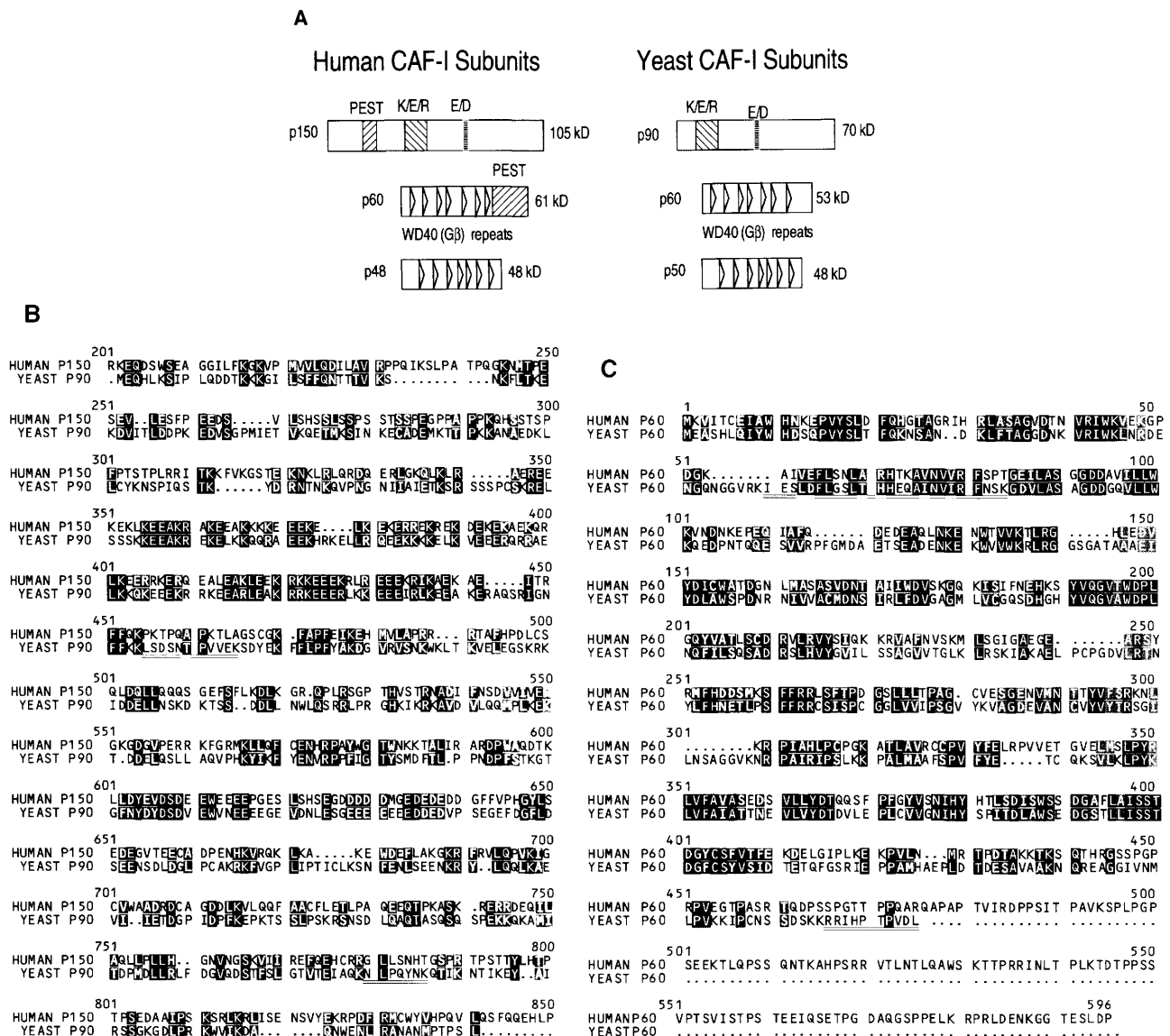


Figure 3. Similarity of yeast CAF-I and human CAF-I subunits. (A) Schematic diagram showing the conserved motifs in the proteins. WD repeats are indicated by triangles. (B) Amino acid sequence alignment of human CAF-I p150 and yeast CAF-I p90 (Cac1p). Numbering indicates residues of the human subunit, starting at residue 201; the entire amino acid sequence of Cac1p is shown. Alignment was made using the GCG PILEUP program. Identical residues are shaded in black; conserved residues are shaded. Groups of conservative residues are defined as follows: (L, A, V, I, F, M, Y) hydrophobic; (F, W, Y) aromatic; (C, S, A, T) small; (K, R, H) basic; (D, E) acidic; (D, N) aspartic; and (E, Q) glutamic. Sequenced Cac1p peptides are underlined. (C) Amino acid sequence alignment of human CAF-I p60 and yeast CAF-I p60 (Cac2p). Shading is as in B. Sequenced Cac2p peptides are double underlined.

that comprise the canonical amino acids that define the repeat itself (Fig. 3C). To date, mutations in the *CAC2* gene (chromosome XIII ORF YML102w) encoding yeast p60 have not been described in the literature, nor has the gene been isolated based on genetic interactions with other mutations.

The yeast genome encodes two genes of the p48 protein family (Verreault et al. 1996) that have now been linked to processes involving histones. One gene has been termed histone acetyltransferase subunit 2 (*HAT2*) (Parthun et al. 1996). This nomenclature reflects the isolation of Hat2p as a protein that copurified with Hat1p,

a B-type histone H4-specific acetyltransferase catalytic subunit (Kleff et al. 1995). The p50 subunit of yeast CAF-I is the other p48 family member, encoded by the *MSI1* gene (chromosome II ORF YBR195c). *MSI1* was isolated as a multicopy suppressor of growth defects in two different screens (Ruggieri et al. 1989; Hubbard et al. 1992), although it is not clear how this is related to CAF-I function (see Discussion). An alignment of the p48 family members from human and yeast has been published previously (Verreault et al. 1996). Although Hat2p has greater similarity to human p48 than Msi1p (35% identical residues for Hat2p compared to 27% for

Msi1p), no peptides derived from Hat2p were obtained in the amino acid sequence analysis of purified yeast CAF-I.

Ultraviolet radiation sensitivity of cells lacking CAF-I

Disruption alleles of each of the genes encoding the yeast CAF-I subunits were constructed, and *cacΔ* strains were isolated. These mutants are viable and do not display growth defects under normal growth conditions. However, mutations in yeast histone H4 can activate a checkpoint that senses DNA damage (Megee et al. 1995), and CAF-I can function during in vitro nucleotide excision repair reactions (Gaillard et al. 1996). Together, these results suggested that proper formation of chromatin may serve to protect the genome from damage. Therefore, we sought to determine whether *cacΔ* strains are more sensitive than isogenic wild-type cells to DNA-damaging agents. Indeed, *cacΔ* strains display increased sensitivity to ultraviolet (UV) radiation; this phenotype cosegregated with the nutritional marker used to identify the disruption allele in all crosses (Fig. 4A; data not shown). Dosage curves demonstrated that disruption of any of the three *CAC* genes results in the same degree of UV sensitivity and that there is no additive effect of combining mutations of any or all three genes (Fig. 4B; data not shown). As a control for the efficiency of the radiation treatment, simultaneously we analyzed cells with a deletion of the *RAD9* gene, one of the checkpoint genes important for delay of the cell cycle in response to DNA damage (Weinert and Hartwell 1988).

UV radiation results in formation of thymine dimers and other photoproducts that are repaired predominantly by nucleotide excision repair enzymes (for review, see Friedberg and Wood 1996). To determine whether the *cacΔ* strains are sensitive to all forms of DNA damage, we also tested sensitivity to gamma-ray irradiation. This treatment produces DNA strand breaks that are repaired in yeast by recombination pathways (Petes et al. 1991; Game 1993). In contrast to their UV sensitivity, *cacΔ* strains are no more sensitive to gamma irradiation than wild-type cells (Fig. 4C). In contrast, cells with a deletion of the *RAD52* gene, required for repair of damage from ionizing radiation (Game and Mortimer 1974), are markedly sensitive to the given radiation doses.

Defects in telomeric silencing in cac mutants

S. cerevisiae has two types of heterochromatic regions in which gene expression is silenced: silent mating loci and telomere-proximal sequences (Gottschling et al. 1990; Dillin and Rine 1995). Chromatin proteins play a role in this silencing. For example, mutations in the histone H3 and H4 amino termini cause derepression of silent loci (Kayne et al. 1988; Johnson et al. 1990; Megee et al. 1990; Park and Szostak 1990). Yeast cells of either a or α mating type that lack CAF-I appear to mate normally (data not shown), indicating that extensive derepression of the normally silent *HML* and *HMR* loci does not occur. Therefore, we tested whether loss of CAF-I would cause derepression of gene expression at telomeric loci.

Derepression of telomere-proximal loci was assayed in strains in which the *URA3* gene has been placed next to a telomere (Gottschling et al. 1990; Aparicio et al. 1991; Aparicio and Gottschling 1994). Derepression of *URA3* expression is then assayed by quantitating the fraction of cells resistant to 5-fluoro-orotic acid (5-FOA), which kills cells expressing the *URA3* gene. A strain with a *URA3*-marked telomere was crossed into *cac1*, *cac2*, and *msi1* mutant strains and tested in this assay. As a positive control, a strain with a *sir2* null allele displayed the expected complete derepression of telomere-proximal *URA3* expression (Table 1; Fig. 5; Aparicio et al. 1991). Significant reduction in the frequency of 5-FOA-resistant cells was also observed in the presence of any of the *cac* null alleles (Table 1; Fig. 5). At a frequency of ~ 1 in 10^5 , FOA-resistant colonies of the same size as observed for wild-type strains were observed in the *cac* mutants. These colonies were fully grown in 2–3 days at 30°C. However, the majority of FOA-resistant *cac* cells were in the form of microcolonies, seen as a faint lawn of cells in the least diluted samples that were best visualized after 5 days of growth (Fig. 5B).

Discussion

We show that all three subunits of the nucleosome assembly factor CAF-I are conserved between yeast and humans. In both of these organisms, CAF-I subunits form complexes that possess similar biochemical functions. Specifically, CAF-I from both yeast (Fig. 1–3) and human cells (Smith and Stillman 1989; Kaufman et al. 1995) acts in vitro to assemble nucleosomes onto replicated DNA. We also note that *Drosophila* CAF-I acts in a similar manner (Kamakaka et al. 1996) and that *Drosophila* CAF-I contains a p48 family member (Tyler et al. 1996). Therefore, CAF-I appears to be conserved functionally and structurally in widely divergent eukaryotic organisms, suggesting an important role in chromosome assembly. Yeast cells lacking the large subunit of CAF-I cannot localize properly the Rap1p protein (Enomoto et al., this issue), a key component of telomeric heterochromatin (Shore 1994). However, cells lacking CAF-I are viable and do not display growth defects under normal conditions, indicating that chromosomes are formed and functional. Furthermore, *cac* mutants do not display dramatic derepression of the silent mating type loci (data not shown; Enomoto et al. this issue), suggesting that defects resulting from an absence of CAF-I may not be uniform throughout the chromosomes. The striking homology of the CAF-I proteins in distant eukaryotes suggests an important and conserved biological role. The observed phenotypes in yeast could imply that CAF-I acts at specific regions of the chromosomes, notably the telomeres (see below). Alternatively, CAF-I may play an auxiliary role at loci other than at telomeres, or it may be redundant with other proteins for most of its functions in vivo. The isolation of the three *CAC* genes will allow for genetic and biochemical approaches to identify other proteins involved in chromosome assembly.

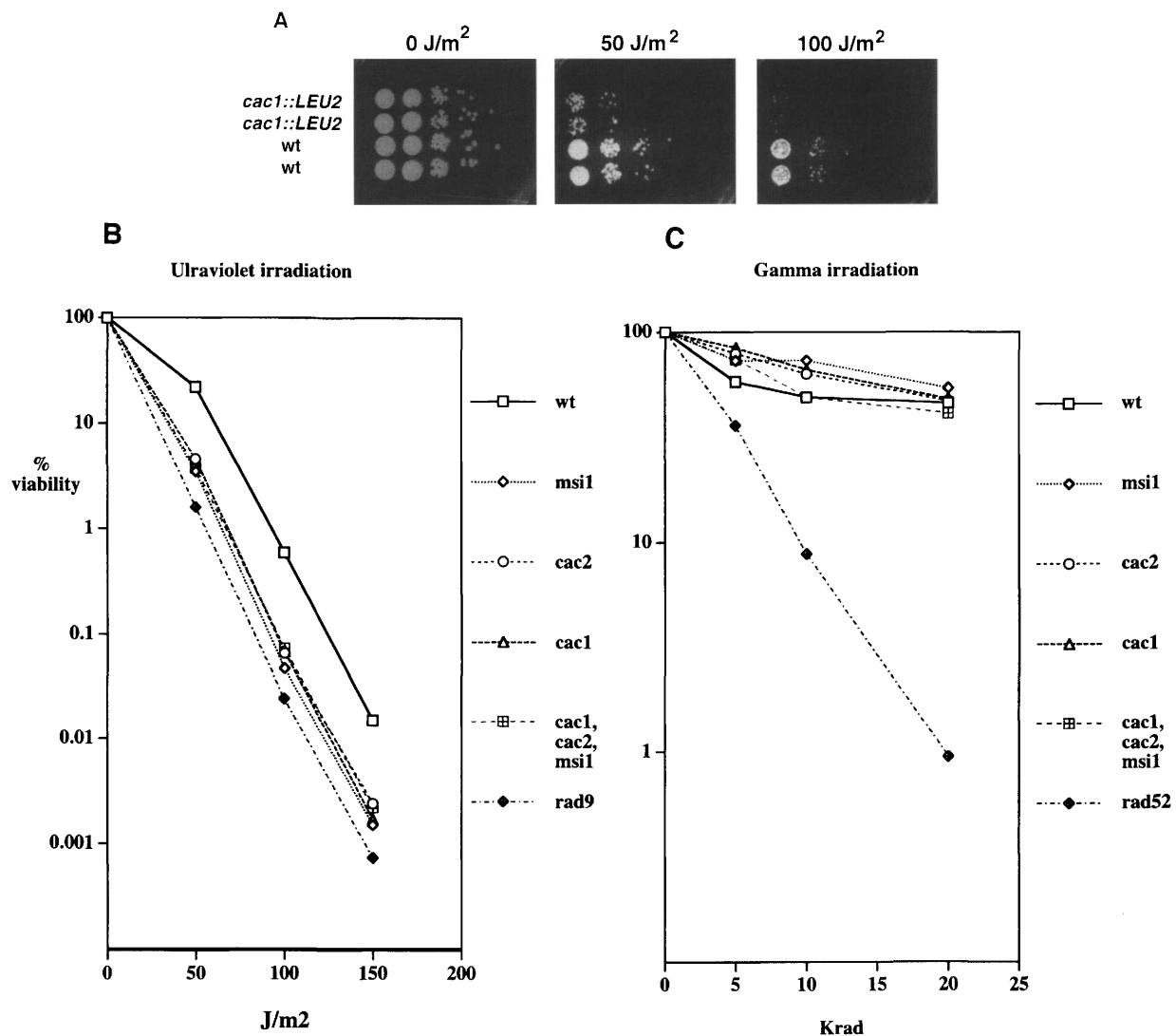


Figure 4. Radiation sensitivity of *cacΔ* mutant strains. (A) Comparison of wild-type and *cacΔ* mutant cells. Four haploid spores were dissected from diploid yeast strain PKY010 (heterozygous for the *cac1::LEU2* allele). Tenfold serial dilutions of log-phase cells were spotted onto a rich medium (YPAD) plate and either not treated (left), exposed to 50 J/m² UV radiation (middle), or 100 J/m² UV (right) before growth for 2 days at 30°C. The upper two spores are Leu⁺; the lower two spores are Leu⁻. (B) UV radiation survival curve. Strains PKY028 (*MATa*; wild-type W303 strain) and isogenic derivatives PKY020 (*cac1::LEU2*), YB0152 (*cac2::TRP1*), YB0145 (*cac3::URA3*), and PKY038 (*cac1::LEU2*; *cac2::TRP1*; *cac3::URA3*) were exposed to the indicated doses of UV light, and viable colonies were counted after growth for 3 days. As a positive control for the efficiency of the irradiation, strain JRY2833 (*MATa*, *his3*, *leu2-3,112*, *ura3-52*, *trp1-289*, *rad9::URA3*) (not isogenic to W303) was irradiated at the same time. (C) Gamma-radiation survival curve. Strains PKY028 (*MATa*; wild-type W303 strain) and isogenic derivatives PKY020 (*cac1::LEU2*), YB0152 (*cac2::TRP1*), YB0145 (*cac3::URA3*), and PKY038 (*cac1::LEU2*; *cac2::TRP1*; *cac3::URA3*) were exposed to the indicated doses of gamma radiation, and viable colonies were counted after growth on YPAD for 3 days. As a positive control for the efficiency of the irradiation, strain BL31-2c (W303 derivative, *MATa*; *rad52::TRP1*; *URA3-ADE2-VIII*) (Li and Lustig 1996) was irradiated at the same time.

Links between DNA synthesis and CAF-I activity

In vitro, a transiently formed structure on replicated DNA stimulates nucleosome assembly by CAF-I (Kamakaka et al. 1996; for review, see Kaufman 1996). Although CAF-I was discovered and characterized using bidirectional SV40 DNA replication reactions (Smith and Stillman 1989; Stillman 1986), recent biochemical experiments show that human CAF-I can function during DNA synthesis reactions initiated by nucleotide ex-

cision repair of DNA damage (Gaillard et al. 1996). We propose that CAF-I is activated by a signal generated during DNA synthesis, regardless of whether the template is undergoing replicative or repair synthesis.

A prediction of this model is that processes requiring DNA synthesis should be affected in cells lacking CAF-I. The availability of *cac* mutants allowed us to test this for the first time. Yeast cells lacking CAF-I display increased sensitivity to UV radiation, but not gamma radiation (Fig. 4). UV causes damage that is chiefly repaired

Table 1. Silencing of a telomere-proximal *URA3* gene in *cac* strains

Strain	Relevant genotype	Large FOA-resistant colonies (3 days)	<i>n</i>	FOA-resistant microcolonies (5 days)	<i>n</i>
PKY090	wt	0.55 ± 0.17	6	N.A.	
PKY106	<i>cac1</i>	<(2.1 ± 1.5) × 10 ⁻⁵ ^a	6	0.011 ± 0.003	4
PKY107	<i>cac2</i>	(5.8 ± 6.5) × 10 ⁻⁵	6	0.012 ± 0.007	4
PKY108	<i>cac1, cac2</i>	(4.2 ± 1.1) × 10 ⁻⁵	4	0.021 ± 0.019	4
PKY116	<i>msi1</i>	<(5.4 ± 2.1) × 10 ⁻⁵ ^b	4	0.042 ± 0.019	4
JRY4470	<i>sir2</i>	<1.8 ± 10 ⁻⁷ ^c	5	N.A.	

The fraction of FOA-resistant cells in a population was determined for these strains as described in Materials and Methods. Colonies of the same size as observed for the wild-type (wt) PKY090 strain were counted after 3 days at 30°C; microcolonies that arise in the *cac* strain were counted after 5 days. The mean number of FOA-resistant colonies ± the standard deviation is shown. (*n*) The number of independent measurements using different cultures. (N.A.) Not applicable; these strains do not form microcolonies on FOA.

^a(<) In one experiment, no large FOA-resistant colonies were observed out of 6.2 × 10⁴ plated.

^b(<) In one experiment, no large FOA-resistant colonies were observed out of 4.1 × 10⁴ plated.

^c(<) In five experiments, no FOA-resistant colonies were observed out of 5.6 × 10⁶ plated.

by nucleotide excision repair, a mechanism that involves DNA synthesis using many of the same proteins involved in bidirectional DNA replication (Friedberg and Wood 1996). In contrast, gamma radiation causes double-strand breaks that are thought to be repaired by mechanisms involving recombination (Petes et al. 1991; Game 1993). As observed in vitro (Gaillard et al. 1996), we propose that DNA synthesis performed during nucleotide excision repair activates CAF-I activity in vivo. In cells lacking CAF-I, either nucleotide excision repair itself may be less efficient, or improper reformation of nucleosomes over repaired regions may lead to reduced viability. In contrast, the observed resistance of *cac* mutants to gamma rays suggests that recombination during strand break repair does not require CAF-I for chromatin reformation.

Another interpretation of the increased UV sensitivity of *cac* mutant cells suggests that DNA could be more susceptible to UV damage because of incomplete chromatin assembly. However, this hypothesis fails to explain why there is not increased sensitivity to all damaging agents.

Reduction of telomeric silencing in *cac* mutants

CAF-I is required for normal levels of the silencing of a telomere-adjacent gene (Table 1; Figure 5; Enomoto et al., this issue). Within a population of *cac* mutant cells, there appear to be at least three different epigenetic states at a telomere. First, the majority of *cac* mutant cells are unable to repress a telomeric *URA3* reporter gene sufficiently to allow growth on FOA (Fig. 5). In addition, there are two distinguishable types of FOA-resistant colonies formed by *cac* strains in these experiments. At a frequency approximately between 10⁻⁵ and 10⁻⁴, these colonies grow as rapidly and attain the same size as observed for a wild-type strain. At much higher frequency (>10⁻²), microcolonies are observed, suggesting that the telomeric *URA3* reporter gene may be subject to incomplete or fluctuating silencing in a subset of the population in the absence of CAF-I. These phenotypes

are best explained at this point by the fact that the *CAC1* gene has also been identified as *RLF2* (Rap1p localization factor-2), which is required for normal telomeric localization of the Rap1p protein (Enomoto et al., this issue). We also note that growth of FOA-resistant cells in a telomere-marked *msi1* mutant strain is qualitatively different; the microcolonies appear larger than observed for the *cac1* and *cac2* strains (Fig. 5; data not shown). Although presently we do not have a mechanistic explanation for this observation, one possibility is that other p48 family members can substitute partially for Msi1p in vivo, resulting in a less severe deficit in telomeric chromatin structure in *msi1* cells. Alternatively, the difference in growth on FOA could result indirectly from a growth advantage unrelated to telomeric chromosome structure (see below).

Therefore, defects in chromosome structure in cells lacking CAF-I have most easily been observed at telomeres. Yeast telomeric DNA acquire single-stranded 3' overhangs even in cells lacking telomerase, the enzyme responsible for synthesis of the telomeric simple-sequence repeats (Wellinger et al. 1996). This result is consistent with proposed mechanisms in which a 5' → 3' nuclease acts to expose single-stranded DNA with a free 3' end (Lingner et al. 1995). The accumulation of single-stranded DNA at telomeres in *cdc13-1* mutants could result from loss of regulation of this putative nuclease (Garvik et al. 1995). Conceivably, there may be multiple rounds of nucleolytic degradation and resynthesis in a dynamic equilibrium process at the telomeres. In turn, this would lead to more frequent utilization of chromatin assembly proteins at telomeres; therefore defects in chromatin assembly would become observed most easily at telomeres. Alternatively, CAF-I may be a specialized chromatin assembly factor responsible mainly for formation of telomeric heterochromatin in yeast.

CAC3 is MSI1

As observed for human and *Drosophila* CAF-I, the p50 subunit of yeast CAF-I is encoded by a member of the

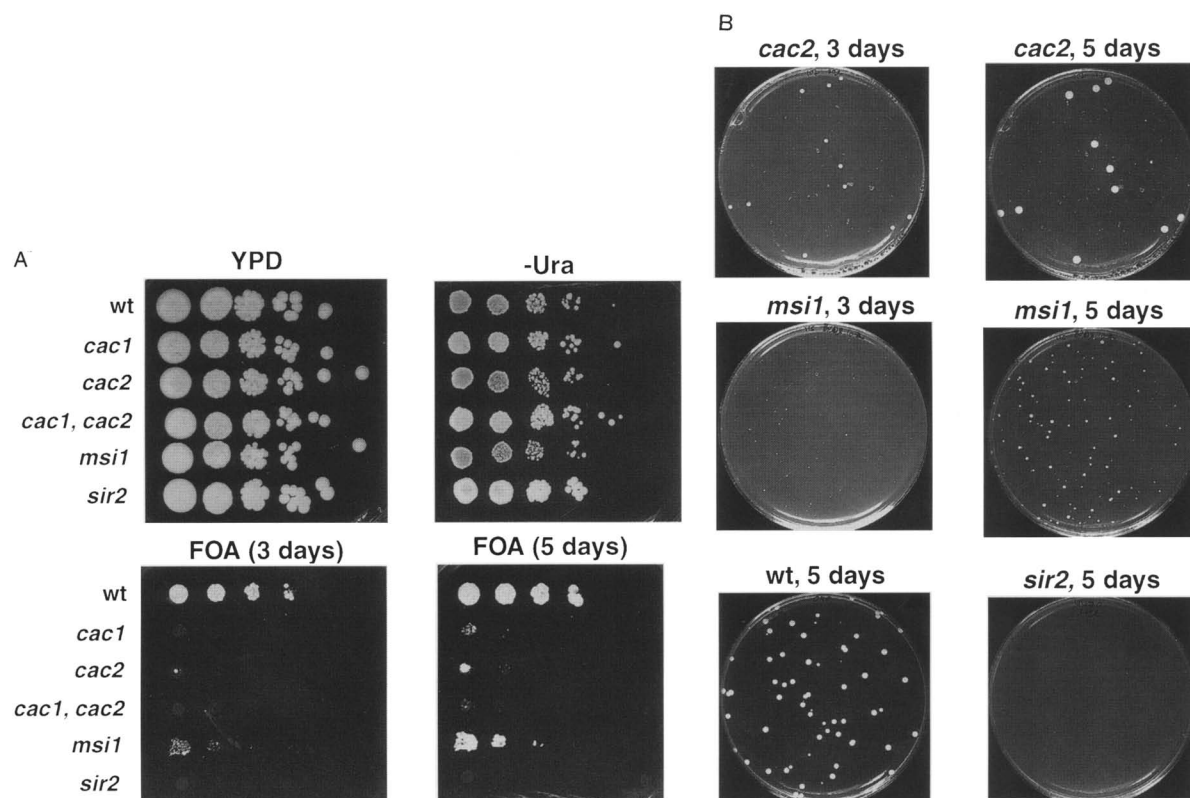


Figure 5. Silencing of a telomeric-proximal *URA3* gene in *cac* mutants. (A) Serial dilution analysis. Strains analyzed were PKY090 (*MATa*; wild-type W303 strain background containing *URA3-VIIL*), and isogenic derivatives PKY106 (*cac1::LEU2*), PKY107 (*cac2::TRP1*), PKY108 (*cac1::LEU2; cac2::TRP1*), PKY116 (*msi1::hisG*), and JRY4470 (*sir2::LEU2*). Tenfold serial dilutions of log-phase cells were plated onto rich medium to determine total cell density (YPD), medium lacking uracil to select for cells with active *URA3* genes (-Ura), or medium containing 5-FOA to select for cells not able to express *URA3* (FOA). The FOA plate was photographed after 3 days and 5 days of growth at 30°C. (B) Colony size of FOA-resistant cells. Cells (6.2×10^4) of strain PKY107 (*cac2, top*) were plated onto an FOA plate and photographed after 3 days (left) and 5 days (right) at 30°C. Both large and small colony sizes are evident after 5 days. Similar sized colonies were observed for PKY106 and PKY108 (see A, and data not shown). Cells (4.1×10^3) of cells of strain PKY116 (*msi1*) were analyzed similarly (bottom). For comparison, 1.7×10^2 cells of wild-type strain PKY090 and 7.6×10^5 cells of *sir2* strain JRY4470 were plated and photographed after 5 days incubation on FOA media.

p48 subfamily of WD repeat proteins; these proteins have been implicated in histone binding and modification (Parthun et al. 1996; Taunton et al. 1996; Tyler et al. 1996; Verreault et al. 1996). The p50 protein isolated as part of yeast CAF-I is encoded by a gene we termed *CAC3*, which has been previously called *MSI1*. The *MSI1* gene had been recovered in two different dosage-suppression screens. Overexpression of the *MSI1* gene suppresses the heat shock sensitivity of *ira1* and activated *RAS2* alleles (thus, the name multicopy suppressor of *IRA*; Ruggieri et al. 1989). The *IRA1* gene encodes a GTPase-activating protein that stimulates the intrinsic GTPase activity of Ras proteins (Ruggieri et al. 1989). *ira1* mutants or constitutively active *RAS2* alleles cause increased intracellular levels of AMP and sensitivity to heat shock. In another screen, Msi1p overexpression suppressed the inability of *snf4* strains (a sucrose nonfermenting strain) to grow on nonfermentable carbon sources (Hubbard et al. 1992). In both of these cases, normal expression levels of *MSI1* did not suppress the mutant phenotype.

It is presently unclear how *MSI1* overexpression suppresses these two distinct phenotypes. One hypothesis is that the role of *MSI1* as part of a CAF-I complex is required for proper deposition of nucleosomes at specific cellular promoters; therefore, overexpression of *MSI1* might lead to titration of chromatin assembly factors and misregulation of a subset of genes (e.g., genes regulating cAMP levels or those required for growth on nonfermentable carbon sources). Another hypothesis, not mutually exclusive of the first, is that Msi1p, like the human p48 subunit (Taunton et al. 1996; Verreault et al. 1996), may exist in a large number of multiprotein complexes, only one of which is CAF-I. In this case, *MSI1* may have a role in processes not directly related to chromatin assembly.

Future experiments are required to determine whether loss or overexpression of *MSI1* affects chromatin structure or expression of specific genes and whether proteins other than CAF-I subunits bind to Msi1p in vivo. We note, however, that loss of any of the three CAF-I subunits results in quantitatively equivalent sensitivity to

UV damage (Fig. 4B), suggesting that it is the three-polypeptide CAF-I protein that is required to protect cells from UV damage.

Materials and methods

Large-scale purification of yeast CAF-I

Whole cell extracts of protease-deficient *S. cerevisiae* strain BJ926 were prepared as described (Bell et al. 1993). Four hundred grams of cells were used per preparation. All steps were carried out at 4°C, and 1 mM DTT, 1 mM PMSF, 1 mM benzamide, 100 µg/ml of bacitracin, and 1 µg/ml of pepstatin were added to all buffers. The initial ammonium sulfate pellet was resuspended in 100 ml of buffer A [25 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.01% NP-40, 10% glycerol], and dialyzed versus 4 liters of buffer A plus 0.1 M NaCl until the conductivity approximated buffer A+0.2 M NaCl. The extract was spun at 10,000 rpm for 15 min in a Sorvall GSA rotor, and the clarified extract (178 ml, 7.5 grams protein) was used for purification. The protein extract (1.9 grams) was loaded onto a 100-ml S-Sepharose column (Pharmacia) equilibrated in buffer A+0.2 M NaCl. Unbound proteins were washed away with nine-column volumes of buffer A+0.2 M NaCl, and bound proteins were eluted with an 800-ml gradient from buffer A+0.2 M to buffer A+0.6 M NaCl.

A 170-ml S-Sepharose fraction centered around buffer A+0.27 M NaCl (~66 mg of protein) was pooled based on conductivity and loaded onto a 0.7-ml Poros HQ20 column equilibrated in buffer I [25 mM imidazole-HCl (pH 6.7), 1 mM EDTA, 0.01% NP-40, 10% glycerol]+0.27 M NaCl using a Pharmacia FPLC system. The Poros HQ column was step-eluted with 5 ml of buffer I+0.4 M NaCl, and remaining bound proteins were eluted with a 10-ml gradient from buffer I+0.4 to buffer I+1.0 M NaCl. Proteins eluting in a broad peak between 0.5 and 0.9 M NaCl were assayed for CAF-I activity (see below), and active fractions were pooled (7.5 ml, 3 mg of protein) and dialyzed versus buffer H [25 mM HEPES-NaOH (pH 8.0), 1 mM EDTA, 0.01% NP-40, 10% glycerol] +0.1 M NaCl. These proteins were loaded onto a 0.4-ml single-strand DNA cellulose column (U.S. Biochemical) equilibrated in buffer H+0.1 M NaCl. Unbound proteins were eluted with 4 ml of buffer H+0.1 M NaCl, and the column was then step-eluted with 3 ml of buffer H+0.3 M NaCl. Eluted proteins (~0.8 mg protein) were loaded onto a 0.3-ml Poros HS column equilibrated in buffer H+0.3 M NaCl, washed with 4 ml of buffer H+0.3 M NaCl, and eluted by an 8-ml gradient from 0.3 to 0.7 M NaCl. Proteins eluting as a broad peak were concentrated by chromatography on a 0.1-ml SP-Sepharose column by binding in buffer H+0.1 M NaCl and were eluted with buffer H+0.5 M NaCl. Concentrated proteins were separated on a 5-ml 15–35% glycerol gradient in buffer A+25 mM NaCl spun at 49,000 rpm for 18 hr in a Beckmann SW55 rotor. Thirteen 350-ml fractions were collected manually from the top of the gradient. Three hundred twenty-five microliters each of fractions 5, 6, and 7 was brought to 20% trichloroacetic acid (TCA), 0.08% sodium deoxycholate (DOC), precipitated, and separated on a preparative SDS–10% polyacrylamide gel.

The p90, p60, and p50 bands were detected by Coomassie blue G staining, excised, digested with *Achromobacter* protease I, and prepared for amino acid sequence analysis as described (Wang et al. 1996). The peptide sequences obtained from p90 and p60 are shown in Figure 3B and 3C, respectively. The peptide sequences obtained from p50 were DITHEASSIPIDLQERYSHWK, LLYDYLNNTNSTK, and PAGNLVGH. These are all encoded by the *MSI1* gene (Ruggieri et al. 1989; Verreault et al. 1996).

Histone-agarose chromatography of yeast CAF-I

Active chromatin assembly factor fractions from the single-strand DNA column were diluted to a conductivity equal to that of buffer A+0.1 M NaCl and loaded onto a 0.15-ml histone-agarose column (Sigma) equilibrated in buffer A+0.1 M NaCl. The column was washed with 1 ml of Buffer A + 0.1 M NaCl and step eluted with 1 ml each of buffer A+0.2 M NaCl, 0.4 M NaCl, and 0.6 M NaCl.

SV40 replication assays and product analyses

Replication reactions were performed and analyzed as described (Kaufman et al. 1995), except that incubation was performed at 30°C for 70–80 min. In some cases (e.g., Fig. 1C,D, lanes 2–4), CAF-I fractions were diluted in buffer A containing 0.1 mg/ml of acetylated BSA, 1 mM DTT, before assay. Products were analyzed by gel electrophoresis in 1% agarose gels.

MNase digestion of replication products was performed as described (Kaufman et al. 1995), with the following modifications: 120 µl of DNA replication-coupled chromatin assembly reactions supplemented with 100 ng of histones H2A/H2B purified from human 293 cells were incubated at 30°C for 80 min. CaCl₂ was then added to 5 mM final concentration, and reactions were divided into 25 µl aliquots before digestion for the indicated amounts of time with 0.2 units of MNase (Sigma) at 20°C. Products were analyzed by gel electrophoresis in 2% agarose gels and autoradiography.

Disruption alleles of the CAC genes

Yeast strains used in this work are listed in Table 2. In all cases, disruption alleles were constructed by single-zygote gene replacement in wild-type diploid W303-1 cells (homozygous for *leu2-3, 112, ura3-1, his3-11,15, trp1-1, ade2-1, can1-100*), and appropriate prototrophic markers were selected using standard genetic techniques (Kaiser et al. 1994). Correct integration was confirmed by Southern blot DNA hybridization. Mutant haploid cells were isolated as prototrophically marked haploids derived from the sporulated diploids.

CAC1 A 5.6-kb *Bam*HI fragment from yeast cosmid 9513 (ATCC) was cloned into the *Bam*HI site of pBSKS+ (Stratagene) to generate plasmid pPK98. The insert is oriented such that the 5' end of the *CAC1* gene is closer to the *Sac*I site of the polylinker. pPK98 was digested with *Bgl*III and *Nhe*I, removing 1.5 kb of the *CAC1* coding sequence, including the ATG start codon. A *Bam*HI–*Xba*I fragment of pJJ283 (Jones and Prakash 1990) containing the *LEU2* gene was inserted into this vector to generate pPK103. pPK103 was digested with *Bam*HI before transformation into W303-1 for generation of the heterozygous *cac1Δ::LEU2* disruption strain PKY010. PKY010 was sporulated to generate the haploid PKY020.

CAC2 A 6.2-kb *Xho*I fragment from yeast cosmid 9514 (ATCC) was cloned into the *Xho*I site of pBSKS+ (Stratagene) to generate plasmid pPK55. The insert is oriented such that the 5' end of the *CAC2* gene is closer to the *Kpn*I site of the polylinker. pPK55 was digested with *Sna*BI and *Sph*I to remove the first 1.1 kb of the *CAC2* gene, including the ATG start codon. A *Sma*I–*Sph*I fragment of pJJ281 (Jones and Prakash 1990) containing the *TRP1* gene was inserted into this fragment to generate pPK57. pPK57 was digested with *Xho*I before transformation into W303-1 for generation of the heterozygous *cac2Δ::TRP1* disruption strain YB0150. YB0150 was sporulated to generate YB0152.

Table 2. *S. cerevisiae* strains used

Strain	Genotype	Reference
W303-1	<i>MATa/MATα</i> ; wild-type W303 strain	
PKY028	<i>MATa</i> ; wild-type W303 strain	
PKY010	<i>MATa/MATα</i> ; <i>cac1::LEU2/CAC1</i>	this work
YB0150	<i>MATa/MATα</i> ; <i>cac2::TRP1/CAC2</i>	this work
YB0149	<i>MATa/MATα</i> ; <i>msi1::URA3/MSI1</i>	this work
PKY018	<i>MATa/MATα</i> ; <i>msi1::hisG-URA3-kan^R-hisG/MSI1</i>	this work
PKY020	<i>MATa</i> ; <i>cac1::LEU2</i>	this work
YB0152	<i>MATa</i> ; <i>cac2::TRP1</i>	this work
YB0145	<i>MATa</i> ; <i>msi1::URA3</i>	this work
PKY041	<i>MATα</i> ; <i>cac1::LEU2</i> ; <i>cac2::TRP1</i>	this work
PKY038	<i>MATa</i> ; <i>cac1::LEU2</i> ; <i>cac2::TRP1</i> ; <i>msi1::URA3</i>	this work
PKY034	<i>MATa</i> ; <i>msi1::hisG-URA3-kan^R-hisG</i>	this work
PKY079	<i>MATa</i> ; <i>msi1::hisG</i>	this work
PKY090	<i>MATa</i> ; <i>URA3-VIII</i>	this work
PKY106	<i>MATa</i> ; <i>URA3-VIII</i> ; <i>cac1::LEU2</i>	this work
PKY107	<i>MATa</i> ; <i>URA3-VIII</i> ; <i>cac2::TRP1</i>	this work
PKY108	<i>MATa</i> ; <i>URA3-VIII</i> ; <i>cac1::LEU2</i> ; <i>cac2::TRP1</i>	this work
PKY116	<i>MATa</i> ; <i>URA3-VIII</i> ; <i>msi1::hisG</i>	this work
BL31-2c	<i>MATa</i> ; <i>rad52::TRP1</i> ; <i>URA3-ADE2-VIII</i>	Li and Lustig (1996)
JRY4470	<i>MATa</i> ; <i>sir2::LEU2</i> ; <i>ura3Δ::LEU2</i> ; <i>TRP1-URA3-VIII</i>	J. Rine (University of California, Berkeley)
JRY2833	<i>MATa</i> ; <i>his3</i> ; <i>leu2-3,112</i> ; <i>ura3-52</i> ; <i>trp1-289</i> ; <i>rad9::URA3</i>	J. Rine

All strains listed are derived from W303-1, except for JRY2833. W303-1 is homozygous for *leu2-3*, *112*; *ura3-1*; *his3-11,15*; *trp1-1*; *ade2-1*; *can1-100*.

CAC3 (MSI1) Plasmid pJH52 carrying the *msi1 Δ ::URA3* allele (Hubbard et al. 1992) was digested with *KpnI* and *SphI* and transformed into wild-type diploid cells to generate the heterozygous disruption strain YB0149. This construct removes the first two-thirds of the *MSI1* coding region, including the ATG start codon. YB0149 was sporulated to generate YB0145.

The telomeric silencing assays required a null allele not marked with *URA3*. A 2.5-kb *BstBI-SalI* fragment from pJH49 containing the *MSI1* gene (Hubbard et al. 1992) was first cloned into *Clal-SalI*-digested pBSKS+ to generate plasmid pPK96. pPK96 was digested with *Clal*, treated with Klenow polymerase to generate a blunt end, and then digested with *BglII*. A 5.4-kb *BamHI-XbaI*/Klenow fragment containing the *URA3* gene and a kanamycin-resistance gene flanked by a direct repeat of bacterial *hisG* DNA was inserted into the prepared pPK96 vector to generate pPK104. A 7.2-kb *BamHI-SalI* fragment of pPK104 was used to transform diploid W303-1 cells to generate the heterozygous disruption strain PKY018. PKY018 was sporulated to generate PKY034. PKY034 was incubated on an FOA plate to select for loss of *URA3* by recombination (as described; Alani et al. 1987) to generate PKY079.

Radiation sensitivity experiments

Log-phase cells (A_{600} ~0.3–0.5) were grown in YPD media (Kaiser et al. 1994) supplemented with 50 μ g/ml of adenine (YPAD), collected by brief centrifugation, washed with water, and resuspended to the original volume in water. Cells were dispersed by sonication with a Branson microtip for five bursts at 40% duty on power setting 3 to prevent clumping. For UV experiments, appropriate dilutions of cells were plated on duplicate YPAD plates and irradiated with a GE germicidal lamp calibrated to deliver 4 J/m² per sec. For gamma-ray experiments, cells in water were exposed to a Cesium-137 source for the indicated

doses and then plated onto duplicate plates. Cells were grown in the dark at 30°C for 3 days before counting; the average of the plate counts is shown. Data are shown from a single preparation of cells, but all the results presented were reproducible in multiple experiments. The *rad9* strain used in Figure 4A is ~10-fold less UV sensitive than a W303-based *rad9* strain isogenic to the *cac* strains used (data not shown).

Measurement of telomeric silencing

Yeast strain PKY028 [*MATa*; wild-type W303 strain] was transformed with a *SalI-NotI* fragment of pADH4UCA-IV (D. Gottschling, pers. comm.) to create a shortened left arm of chromosome VII with the *URA3* gene adjacent to the telomere as described (Gottschling et al. 1990). Correct integration was confirmed in strain PKY090 by DNA hybridization of Ura⁺ transformants with sequences from the truncated *ADH4* gene (data not shown). PKY090 was crossed to PKY041 [*MATa*, *cac1::LEU2*; *cac2::TRP1*] to generate the other strains tested. Log-phase cells (A_{600} ~0.3–0.6) were prepared as described for the radiation sensitivity experiments. Cells were serially diluted in water, and 5- μ l spots were placed on the indicated media and grown at 30°C for 2 days. Alternatively, appropriate dilutions of cells were plated onto either YPAD medium to determine total cell number or on FOA medium and grown at 30°C. Large cells were counted at 3 days, and microcolonies were counted after 5 days.

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