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Heterochromatin Dynamics in Mouse Cells: Interaction between Chromatin Assembly Factor 1 and HP1 Proteins

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Summary

Mechanisms contributing to the maintenance of heterochromatin in proliferating cells are poorly understood. We demonstrate that chromatin assembly factor 1 (CAF-1) binds to mouse HP1 proteins via an N-terminal domain of its p150 subunit, a domain dispensable for nucleosome assembly during DNA replication. Mutations in p150 prevent association with HP1 in heterochromatin in cells that are not in S phase and the formation of CAF-1-HP1 complexes in nascent chromatin during DNA replication in vitro. We suggest that CAF-1 p150 has a heterochromatin-specific function distinct from its nucleosome assembly function during S phase. Just before mitosis, CAF-1 p150 and some HP1 progressively dissociate from heterochromatin concomitant with histone H3 phosphorylation. The HP1 proteins reassociate with chromatin at the end of mitosis, as histone H3 is dephosphorylated.

Introduction

Heterochromatin was originally defined through cytological studies as that fraction of the genome that remains visibly condensed throughout the mitotic cell cycle. More recently, the term heterochromatin has also been used to describe a number of functionally specialized forms of chromatin that are structurally and biochemically distinct. For instance, Sir3p and Sir4p, which are key structural proteins in the relatively well-defined heterochromatin that forms over the telomeres and the silent mating type loci of *S. cerevisiae* (Grunstein, 1998), do not have any obvious ortholog in other eukaryotes. Similarly, heterochromatin-binding protein 1 (HP1), which is a structural component of pericentric heterochromatin in a number of widely divergent species (*S. pombe*, *D. melanogaster*, mice, and humans [James et al., 1989; Singh and Klar, 1992; Ekwall et al., 1995]), does not exist in *S. cerevisiae*.

In spite of these important differences, a number of similarities also exist between these distinct forms of heterochromatin. Heterochromatin generally replicates late during S phase, has a low gene density, and contains large blocks of repetitive DNA, such as those found in the pericentric regions of chromosomes. Heterochromatin is also packaged into a structure that is relatively inaccessible to DNA-modifying reagents (Singh and Klar, 1992; Elgin, 1996).

With the notable exception of a few endogenous genes (Elgin, 1996), genes artificially introduced or translocated into heterochromatin are subject to transcriptional silencing (reviewed in Singh and Klar, 1992; Elgin, 1996; Grunstein, 1998). However, genes that are silenced by packaging into heterochromatin are usually expressed in a small fraction of cells in a population. These two states of gene expression in genetically identical cells are maintained by an epigenetic "imprint" that can be stably propagated through mitosis and, remarkably, even through meiosis (Grewal and Klar, 1996). The integrity of heterochromatin structure and the silencing of heterochromatic genes are dependent upon both the maintenance of histones in a hypoacetylated state (Ekwall et al., 1997; Grewal et al., 1998) and the presence of a number of additional nonhistone proteins such as the HP1 and Sir proteins (Elgin, 1996; Grunstein, 1998).

In *Drosophila*, HP1 is a component of both pericentric (James et al., 1989; Pak et al., 1997) and telomeric heterochromatin (James et al., 1989; Fanti et al., 1998). Loss-of-function mutations in the HP1 gene result in a number of structural defects in mitotic chromosomes including a lack of chromatin condensation (Kellum and Alberts, 1995) and telomere fusions that often generate chromosome breaks (Fanti et al., 1998). Mutations in *S. pombe* Swi6p, which is structurally similar to HP1, result in a high rate of chromosome loss and an elevated incidence of lagging chromosomes during anaphase (Ekwall et al., 1995). HP1 does not appear to bind to DNA directly in *Drosophila*. Instead, the localization of HP1 to pericentric heterochromatin is dependent upon binding to the origin recognition complex (ORC; Huang et al., 1998). Although ORC was originally identified as a protein involved in initiation of DNA replication (Bell and Stillman, 1992), it was subsequently found to play an independent role in heterochromatin structure and function in both *S. cerevisiae* and *Drosophila* (Dillin and Rine, 1997; Pak et al., 1997; Huang et al., 1998). The protein(s) that anchor HP1 to telomeres, if any, are not known.

Chromatin assembly factor 1 (CAF-1) comprises three polypeptide subunits known as p150, p60, and p48 (Cac1p, Cac2p, and Cac3p in *S. cerevisiae*). CAF-1 deposits newly synthesized and acetylated histones H3/H4 into nascent chromatin during DNA replication (Smith and Stillman, 1991; Kaufman et al., 1995). At least in human cells, CAF-1 is a fairly general nucleosome assembly factor that is targeted to both euchromatic and heterochromatic DNA replication foci (Krude, 1995). In *S. cerevisiae*, deletion of the genes encoding any of the

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Table 1. Mouse TIF1β, CAF-1 p150, and a Novel Protein of Unknown Function Were Identified in a Two-Hybrid Screen for Proteins Interacting with MOD1

LEXA DBD Fusions	VP16 Activation Domain (AD) Fusion Proteins							
	VP16 AD Only		VP16 AD–TIF1β		VP16 AD–Unknown		VP16AD–p150	
	His	Colony	His	Colony	His	Colony	His	Colony
MOD1	−	white	+	blue	+	blue	+	blue
MOD1-C	−	white	+	blue	+	blue	+	blue
Lamin	−	white	−	white	−	white	−	white
Daughterless	−	white	−	white	−	white	−	white
LexA only	−	white	−	white	−	white	−	white

The yeast reporter strain was transformed with the indicated plasmids. To assay for histidine prototrophy, transformants were grown on synthetic medium plates lacking histidine and incubated at 30°C for 3 days. β-galactosidase activity was determined qualitatively using a filter assay. In this assay, positive colonies became blue in 15–30 min, whereas negative colonies remained white after overnight incubation. MOD1, full-length MOD1; MOD1-C, shadow chromodomain of MOD1 (amino acid residues 104–185).

three subunits of the CAF-1 protein (*cac1*Δ, *cac2*Δ, or *cac3*Δ) results in a silencing defect of reporter genes packaged into telomeric heterochromatin (Enomoto et al., 1997; Kaufman et al., 1997). Because *S. cerevisiae* heterochromatin is biochemically very different from that of other eukaryotes, it is not clear whether the role played by *S. cerevisiae* CAF-1 in the epigenetic inheritance of heterochromatin structures is evolutionarily conserved.

This paper describes an association between the large subunit of CAF-1 (p150) and both MOD1 and HP1α, two of the three HP1 family members in the mouse. The p150 subunit of CAF-1 binds to HP1 proteins directly through an amino-terminal sequence, termed MIR for MOD1 interaction region, which is also present in a number of other proteins that bind to MOD1. Mutations of the MIR prevent p150 from binding to HP1 proteins and accumulating into heterochromatin in mouse cells that are not in S phase. However, these mutations do not affect p150-mediated de novo nucleosome assembly in vitro or p150 targeting to both euchromatic and heterochromatic DNA replication foci in vivo. These results argue that, through its ability to bind to HP1 proteins, the N-terminal domain of CAF-1 p150 plays a role in the maintenance of heterochromatin that is distinct from the role of p150 in nucleosome assembly during S phase. We also demonstrate a dramatic dissociation of both MOD1 and CAF-1 p150 from heterochromatin as cells prepare for mitosis. This gradual dissociation occurs in parallel with histone H3 phosphorylation and well before the visible onset of mitosis. In late anaphase or telophase, MOD1 rebinds to mitotic chromosomes in parallel with H3 dephosphorylation.

Results

Identification of Proteins Interacting with the Shadow Chromodomain of MOD1

A dual reporter yeast two-hybrid screen was carried out to identify proteins that interact with mouse MOD1 (Table 1). The plasmids obtained in the two-hybrid screen were sequenced and found to encode three distinct mouse polypeptides, each represented by multiple overlapping cDNA clones. Fusion of the proteins encoded by each of these cDNA clones to the VP16 activation domain induced transcription of both reporter genes (*HIS3* or *lacZ*) when either full-length MOD1 or its carboxy-terminal domain, but not unrelated proteins such as lamin or daughterless, were fused to the LexA DNA-binding domain (Table 1).

The first group of cDNA clones encoded the transcriptional intermediary factor 1β (TIF1β). TIF1α and β are sequence-related proteins that were both previously reported to bind to the mouse HP1α and MOD1 proteins (Remboutsika et al., 1999, and references therein). TIF1α associates in a ligand-dependent manner with several nuclear hormone receptors. TIF1β does not bind to nuclear hormone receptors but promotes transcriptional repression mediated by the KRAB domain of KOX1. Although both TIF1α and β can repress transcription of reporter genes when fused to heterologous DNA-binding domains, the precise function of their interaction with HP1 proteins is not clear (Remboutsika et al., 1999). The second group of cDNA clones encoded a novel protein of unknown function represented by two EST cDNA clones (GenBank accession numbers AA153281 and AA003533). The third group of cDNA clones encoded a protein with similarity to the p150 subunit of human CAF-1. Comparison of the amino acid sequences predicted from a large number of overlapping cDNA clones isolated in the two-hybrid screen revealed the existence of a conserved amino acid sequence (Figure 1A). This region, MIR, was present in the three types of proteins isolated in our two-hybrid screen, as well as in TIF1α and human CAF-1 p150 (Figure 1A).

To demonstrate that the interaction between MOD1 and the MIR-containing proteins was direct and to investigate which region of MOD1 mediated the interaction, we expressed and purified the MIR peptides as GST fusions in *E. coli*. The GST–MIR fusion proteins were used in pulldown assays with recombinant MOD1 purified from *E. coli*. As shown in Figure 1B, both full-length MOD1 and the MOD1 shadow chromodomain directly bound to a GST fusion protein containing the CAF-1 p150 MIR (amino acid residues 176–327 of mouse p150) (Figure 1B, lanes 2 and 6). In contrast, the chromodomain of MOD1 did not bind to the p150 MIR (Figure 1B, lane 4). The shadow chromodomain was therefore necessary and sufficient for the interaction. We obtained essentially the same results with GST–MIR fusions derived from the other proteins isolated in the two-hybrid screen, namely TIF1β and the novel protein of unknown

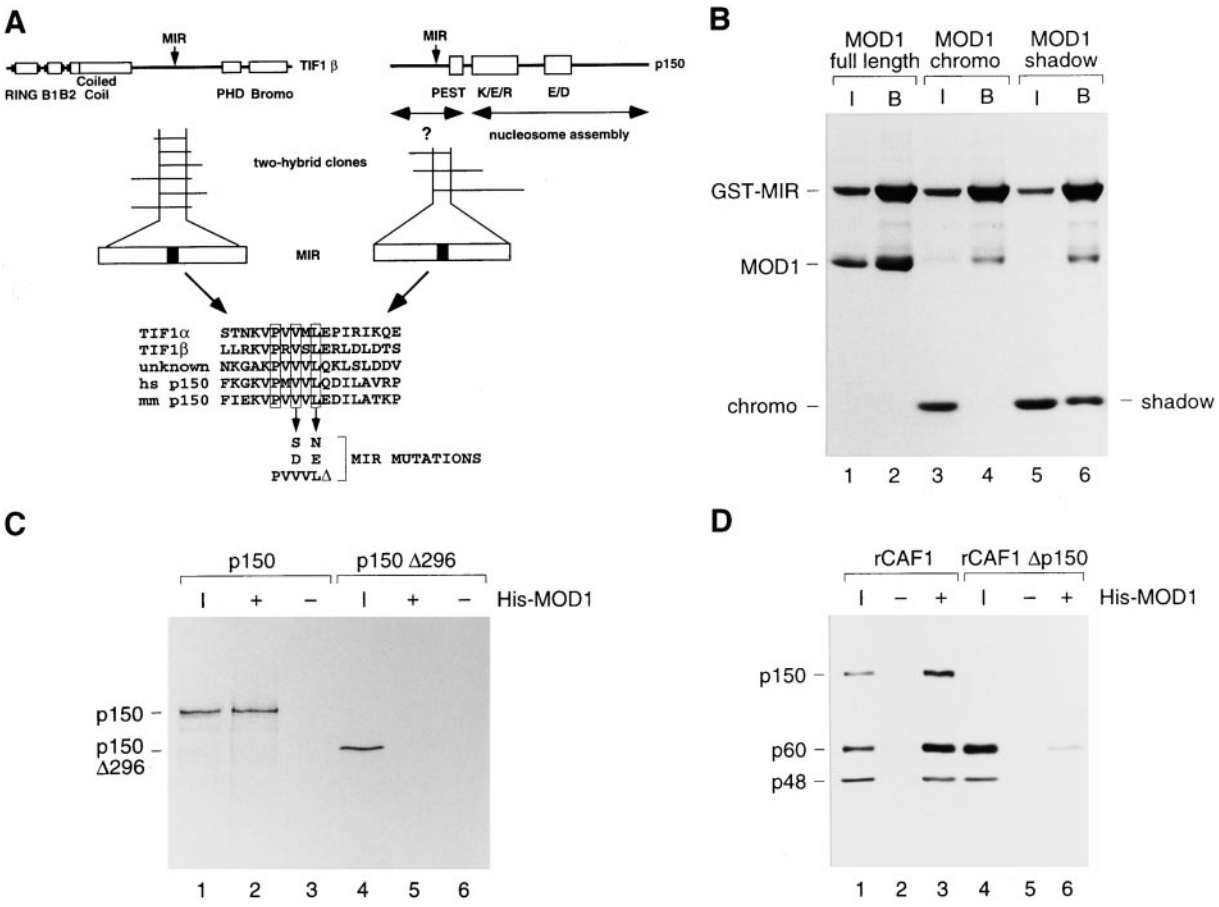

Figure 1. A MIR within the N-Terminal Domain of CAF-1 p150 Binds to the Shadow Chromodomain of MOD1

(A) A number of overlapping cDNA clones encoding portions of mouse TIF1β and CAF-1 p150 were isolated through a two-hybrid screen using MOD1 as bait. This information, along with peptide binding studies (data not shown), was used to identify a conserved motif, present in TIF1α, TIF1β, CAF-1 p150, and a novel mouse protein of unknown function. Site-directed point mutations and a short deletion of conserved MIR residues are shown.

(B) The shadow chromodomain of MOD1 is necessary and sufficient for binding to the p150 MIR (residues 176–327 of mouse CAF-1 p150). Pulldown assays were performed by mixing purified full-length MOD1 (lanes 1 and 2), MOD1 chromodomain (lanes 3 and 4), or MOD1 shadow chromodomain (lanes 5 and 6) with recombinant GST–p150 MIR immobilized on glutathione agarose beads. After extensive washes, the proteins that remained bound to beads were detected by SDS-PAGE and Coomassie blue staining (lanes labeled B). Lanes labeled I show the amounts of GST–MIR and MOD1 peptides equivalent to 40% of the input protein.

(C) MOD1 binds to CAF-1 primarily through the p150 N-terminal domain. In vitro translated full-length human p150 (lanes 1–3) or p150 lacking the N-terminal domain (lanes 4–6) were incubated with nickel-NTA-agarose beads either without (lanes 3 and 6) or with (lanes 2 and 5) an excess of His-tagged MOD1 protein. After extensive washes, the proteins that remained bound to beads were detected by SDS-PAGE and fluorography (lanes 2, 3, 5, and 6). Lanes labeled I (1 and 4) contain the equivalent of 50% of the input protein.

(D) Purified recombinant CAF-1 (lanes 1–3) or CAF-1 lacking the p150 subunit (lanes 4–6) were incubated with nickel-NTA-agarose beads either without (lanes 2 and 5) or with (lanes 3 and 6) an excess of His-tagged MOD1. After extensive washes, the CAF-1 subunits that remained bound to beads were detected by SDS-PAGE and Western blotting (lanes 2, 3, 5, and 6). Lanes labeled I (1 and 4) contain the equivalent of 50% of the input protein.

function (data not shown). The three distinct MIRs had no obvious homology except in a short peptide motif (Figure 1A), suggesting that this peptide provided a key component to the interaction with MOD1. In addition to MOD1, we found that the p150 MIR also bound directly to MOD2 and HP1α, the two other HP1 family members in the mouse (data not shown).

Mouse and Human CAF-1 p150 Are MOD1-Binding Proteins

A mouse library was screened to isolate a full-length cDNA (GenBank accession number AJ132771) that contained sequences identified in the two-hybrid screen. Overall, the mouse and human p150 polypeptides exhibited 69% identity and 75% similarity to each other. This low sequence similarity was surprising. However, TBLASTN searches of the mouse EST database using either human or mouse p150 as query sequences only returned EST clones that were identical to portions of the full-length mouse cDNA, arguing that there is one predominant p150 cDNA in the mouse. Most of the amino acid similarity between the mouse and human p150 lies within the K/E/R, E/D, and C-terminal p60-binding domains, three regions that are essential for nucleosome assembly (Figure 1A; Kaufman et al., 1995). The N-terminal domains of mouse and human p150 are

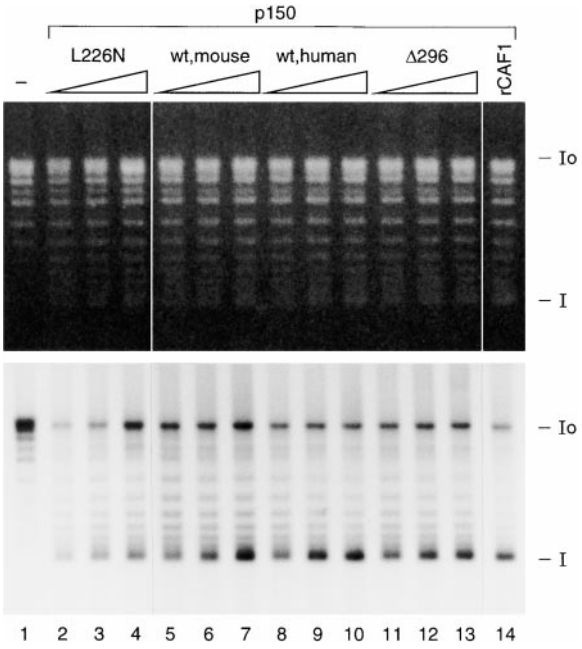

Figure 2. MIR Mutations Do Not Impair the Ability of p150 to Promote Nucleosome Assembly during DNA Replication In Vitro

In vitro translated wild-type and mutant CAF-1 p150 were assayed for their ability to promote nucleosome assembly during SV40 DNA replication. ^{32}P-labeled DNA replication products were analyzed by agarose gel electrophoresis followed by ethidium bromide staining (top) and autoradiography (bottom). For each protein, three assays were performed, corresponding to 2-fold increases in protein concentration in each lane. Lanes 2–4, mouse CAF-1 p150 L226N; lanes 5–7, wild-type mouse p150; lanes 8–10, wild-type human p150; lanes 11–13, human p150 lacking amino acids 1–296. Negative and positive controls containing either no protein (lane 1) or recombinant CAF-1 (lane 14) are also shown. The positions of plasmid DNA form Io (topologically relaxed) and form I (supercoiled DNA) are indicated. The slightly weaker activity of the L226N mutant is most likely due to the lower amount of this protein that is produced by in vitro translation in the rabbit reticulocyte lysate.

more divergent (51% identity and 58% similarity between amino acids 1–296 of human and mouse p150). Intriguingly, the MOD1 interaction region lies within this relatively less conserved N-terminal domain of p150 (Figure 1A).

The N-terminal domain of human p150 (amino acids 1–296) was essential for binding to MOD1 (Figure 1C). In addition, recombinant human CAF-1 lacking the p150 subunit bound much more weakly to MOD1 than CAF-1 containing the three intact subunits (Figure 1D). Taken together, these data argued that the native CAF-1 protein directly bound to MOD1 and that this interaction was, to a large extent, mediated through the MIR present within the N-terminal domain of mouse and human p150. Moreover, the location of the MIR within the amino-terminal domain of CAF-1 p150, a region dispensable for nucleosome assembly (Figure 2), suggested that the MIR had a function that was distinct from the role of CAF-1 in nucleosome assembly.

There are three hydrophobic residues that are absolutely conserved among the MIR sequences of five distinct MOD1-binding proteins (Figure 1A). In order to investigate the function of the interaction between CAF-1 and MOD1, we made point mutations that disrupted the hydrophobic character of the MIR (Figure 1A) and decreased the ability of mouse CAF-1 p150 to bind to MOD1 and HP1α in vitro (data not shown) and in vivo (Figure 5B). The five mutants we made (four point mutations and a deletion of five contiguous hydrophobic amino acids; Figure 1A) behaved similarly in the assays described in this paper.

Wild-type and MIR mutants of CAF-1 p150 were expressed by in vitro translation in a rabbit reticulocyte lysate. Similar amounts of each ^{35}S-labeled protein were assayed for their ability to promote nucleosome assembly during simian virus 40 (SV40) DNA replication in a human S100 extract. As previously reported (Kaufman et al., 1995), human p150 is sufficient to promote nucleosome assembly in this system, because the other two subunits of CAF-1 (p60 and p48) and newly synthesized histones are present in the S100 extract (Figure 2, lanes 8–10). The p150 subunit of mouse CAF-1 also promoted nucleosome assembly (Figure 2, lanes 5–7), indicating that mouse p150 can bind to the other subunits of the CAF-1 protein present in the human S100 extract and form a protein that is active in nucleosome assembly. Thus, in spite of the lack of similarity between their amino-terminal domains, mouse p150 can substitute for human p150 to promote nucleosome assembly in vitro. Point mutation (Figure 2, lanes 2–4) or deletion of the MIR (data not shown) did not impair the nucleosome assembly activity of p150. This result was expected because deletion of the entire N-terminal domain of human p150 (amino acids 1–296) did not prevent nucleosome assembly (Figure 2, lanes 11–13; Kaufman et al., 1995).

Formation of a Complex between MOD1 and CAF-1 p150 in Nascent Chromatin Is Dependent upon the N-Terminal Domain of p150

Heterochromatin maintenance in proliferating cells not only requires the formation of nucleosomes, but also the synthesis and regulated assembly of a number of nonhistone proteins, such as the HP1 proteins, that must be specifically targeted to heterochromatin. The fact that the N-terminal domain of p150 was necessary and sufficient for MOD1 binding, yet dispensable for nucleosome assembly, raised the possibility that CAF-1 may be able to bind to both histones H3/H4 and MOD1 simultaneously and deliver them to nascent heterochromatin during DNA replication.

The ability of CAF-1 to promote incorporation of MOD1 into nascent chromatin was tested using a cell-free system for SV40 DNA replication. Although the chromatin formed in this system is certainly very different from native heterochromatin, we used this cell-free system to determine whether CAF-1 and MOD1 can remain associated with nascent chromatin formed in vitro. Interestingly, even a large molar excess of MOD1 over CAF-1 did not interfere with the ability of CAF-1 to promote nucleosome assembly during DNA replication in vitro (Figure 3A, lanes 1–3). Following DNA replication and nucleosome assembly, nascent chromatin formed in the presence of either CAF-1 alone or a CAF-1-MOD1 complex were immunoprecipitated with antibodies against either acetylated histone H4 or CAF-1 (Figure 3A, lanes 5, 7, and 8). However, nascent chromatin could only

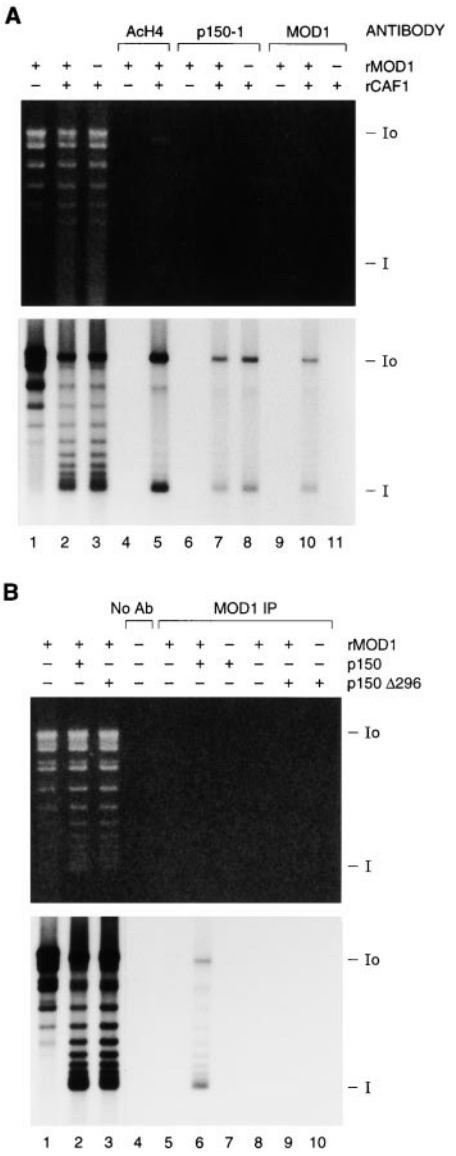

Figure 3. CAF-1 p150 and MOD1 Remain Associated with Nascent Chromatin Formed during DNA Replication In Vitro

(A) Nucleosome assembly reactions were performed in the presence of additional MOD1 and/or CAF-1. ^{32}P-labeled DNA replication products were either analyzed directly (lanes 1–3) or following immunoprecipitation with antibodies against acetylated H4 (AcH4), p150, or MOD1.

(B) Nucleosome assembly reactions were performed with in vitro translated wild-type human p150 or p150 lacking amino acids 1–296 with or without recombinant MOD1. DNA replication products were either analyzed directly (lanes 1–3) or following immunoprecipitation with MOD1 antibodies (lanes 5–10) or a mock precipitation without antibody (lane 4).

be immunoprecipitated by MOD1 antibodies when both CAF-1 and MOD1 were added during DNA replication (Figure 3A, lanes 9–11). These experiments revealed that a small fraction of the CAF-1 and MOD1 molecules in the nucleosome assembly reaction remained associated with the nascent chromatin formed during DNA replication.

A number of controls were performed to rule out the possibility that the presence of radiolabeled DNA in these immunoprecipitates was due to nonspecific binding of the nascent chromatin to the antibody beads. Immunoprecipitation of replicated DNA by the acetylated histone H4, p150, or MOD1 antibody beads required the presence of CAF-1 (Figure 3A, lanes 4, 6, and 9). In addition, this immunoprecipitation was specific for the replicated DNA, because the bulk of the input DNA, which did not replicate and did not get packaged into chromatin under these conditions, was not immunoprecipitated by any of the antibodies used (Figure 3A, ethidium stain). Finally, the MOD1 antibody did not bind nonspecifically to nascent chromatin because this antibody did not bring down radiolabeled DNA when nascent chromatin was formed by CAF-1 in the absence of MOD1 (Figure 3A, lane 11).

The association of MOD1 with nascent chromatin described in Figure 3A could be mediated by an interaction with either histones or the small fraction of CAF-1 p150 that remained bound to the nascent chromatin. To discriminate between these two possibilities, nucleosome assembly reactions were performed with MOD1 and either wild-type or p150Δ296, a deletion of the entire N-terminal domain of p150 that abolished its binding to MOD1 (Figure 1C). Although both p150 and p150Δ296 supported DNA replication–dependent nucleosome assembly equally well (Figure 3B, lanes 1–3), only wild-type p150 promoted incorporation of MOD1 into nascent chromatin (Figure 3B, lanes 6 and 9). Controls in which p150, p150Δ296, or MOD1 were omitted from the reactions revealed that the immunoprecipitation of radiolabeled DNA was not due to nonspecific binding of the replicated DNA to the MOD1 antibody beads (Figure 3B, lanes 5, 7, 8, and 10). Thus, the association of MOD1 with nascent chromatin required the N-terminal domain of p150 molecules that remained part of the chromatin formed during DNA replication. This result also showed that CAF-1 p150 and MOD1 can coexist in nascent chromatin and suggested that CAF-1 may contribute to heterochromatin maintenance in proliferating cells by bringing newly synthesized HP1 proteins to heterochromatic DNA replication foci.

Targeting MOD1 to Heterochromatin Does Not Require Heterochromatin Replication

To test the hypothesis that heterochromatin DNA replication was required for targeting newly synthesized HP1 proteins to heterochromatin, MOD1 was transiently expressed as a green fluorescent protein (GFP) fusion from a mouse histone H3 promoter, to force expression of GFP–MOD1 during early S phase. After 12 hr in the presence or absence of inhibitors of DNA synthesis, targeting of GFP–MOD1 to heterochromatin was assayed by immunofluorescence. As shown in Figure 4A, most cells treated with hydroxyurea arrested early in S phase, prior to heterochromatin replication, as revealed by the large number of small foci containing the proliferating cell nuclear antigen (PCNA). At this stage of S phase, the p150 subunit of CAF-1 colocalized with PCNA in euchromatic DNA replication foci (Krude, 1995; Shibahara and Stillman, 1999; Figure 6). However, in these cells, GFP–MOD1 was clearly targeted to heterochromatin and was resistant to Triton X-100 extraction, as is

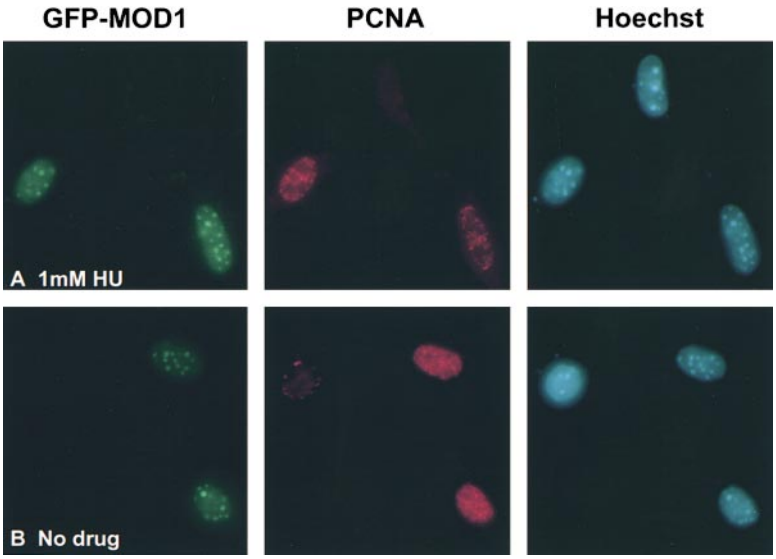

Figure 4. GFP–MOD1 Expressed Early during S Phase Is Targeted to Heterochromatin in the Absence of Heterochromatin Replication

Mouse NIH 3T3 cells were transiently transfected with a construct expressing GFP–MOD1 from the histone H3 promoter. The cells were incubated for 12 hr either with (A) or without 1 mM hydroxyurea (B), permeabilized with Triton, fixed with formaldehyde, and analyzed by immunofluorescence microscopy with PCNA antibodies to localize DNA replication foci (red). To highlight the location of heterochromatin, the DNA was stained with limiting amounts of Hoechst 33258 (cyan), which preferentially intercalates into AT-rich DNA in pericentric heterochromatin.

the case for endogenous MOD1. The same was true when aphidicolin was used instead of hydroxyurea (data not shown) or in early S phase cells that were not treated with drug (Figure 4B). Thus, passage through late S phase and heterochromatin replication were not required for MOD1 targeting to heterochromatin. However, it was not clear whether the GFP–MOD1 expressed in these early S phase cells was properly packaged into heterochromatin.

CAF-1 p150 MIR Mutations Prevent Binding to HP1 Proteins In Vivo

To address the in vivo relevance of the binding of CAF-1 p150 to MOD1, we made constructs for transient expression of GFP fused to either wild-type p150 or the MIR mutants described in Figure 1A. As shown in Figure 5A, expression of wild-type p150 as a GFP fusion protein in mouse cells resulted in the accumulation of GFP–p150 in heterochromatin and its colocalization with endogenous MOD1 or HP1α (Figure 5A, upper panel, and data not shown). Several lines of evidence argued that this result was not merely due to overexpression of GFP–p150. First, although individual transfected cells expressed GFP–p150 over a very broad concentration range, colocalization with HP1 proteins was independent of the level of expression of GFP–p150 (data not shown). Second, this colocalization was also seen in stably transfected cells, where GFP–p150 was expressed at a much lower level than in transient transfection assays (data not shown). Third, the same result was also obtained upon transient expression of human p150 into mouse cells, without any GFP moiety, where the protein was detected using a monoclonal antibody (mAb p150–1) that specifically recognized human, but not mouse p150 (data not shown). Fourth, point mutations of conserved MIR residues abolished the association of p150 with heterochromatin in vivo (Figure 5A, lower panel). This last result was observed with all the MIR mutants described in Figure 1A and argued that the conserved residues within MIR were critical for CAF-1 p150 to remain stably bound to HP1 proteins in heterochromatin in vivo.

Consistent with the colocalization of GFP–p150 with MOD1, a small fraction of endogenous p150 and GFP-wild-type p150 were coimmunoprecipitated by HP1α antibodies (Figure 5B, lanes 1 and 2). In contrast, deletion or point mutation of conserved MIR residues completely abolished coprecipitation of GFP–p150 with HP1α (Figure 5B, lanes 3–6). This result showed that the MIR was crucial for p150 binding to HP1α. The relatively small fraction of p150 that was coprecipitated with HP1α in these experiments (2%–4%) was an underestimate because only a small fraction of the HP1α present in the extract was immunoprecipitated (data not shown). Although we only found a weak coprecipitation of p150 with MOD1 antibodies (data not shown), the fraction of MOD1 precipitated by these antibodies was also very small. In addition, we found that a significant fraction of both p150 and HP1 proteins were very difficult to extract from mouse cell nuclei, being resistant to both high salt and nonionic detergent extractions. Coimmunoprecipitation of CAF-1 p150 was resistant to 100 μg/ml ethidium bromide, indicating that the association of p150 with HP1α was not indirectly mediated by nucleic acids present in the nuclear extract. The mouse and human CAF-1 p150 subunits contain PEST motifs (Kaufman et al., 1995) characteristic of proteins that undergo rapid turnover in vivo. Consistent with this, a number of polypeptides smaller than p150 could be detected in nuclear extracts of L cells. Some of these polypeptides were breakdown products of p150 because they were coimmunoprecipitated by HP1α antibodies (Figure 5B). However, a number of these were not coimmunoprecipitated, suggesting that they were either breakdown products of p150 that were not bound to HP1α or unrelated proteins.

The MIR Is Not Required for CAF-1 Recruitment to DNA Replication Foci

In a small fraction of transfected cells, GFP–p150 did not colocalize with endogenous HP1 proteins, but instead appeared in small foci that were shown to coincide with euchromatic DNA replication foci in early S phase cells (see below). During S phase in human cells, CAF-1 is

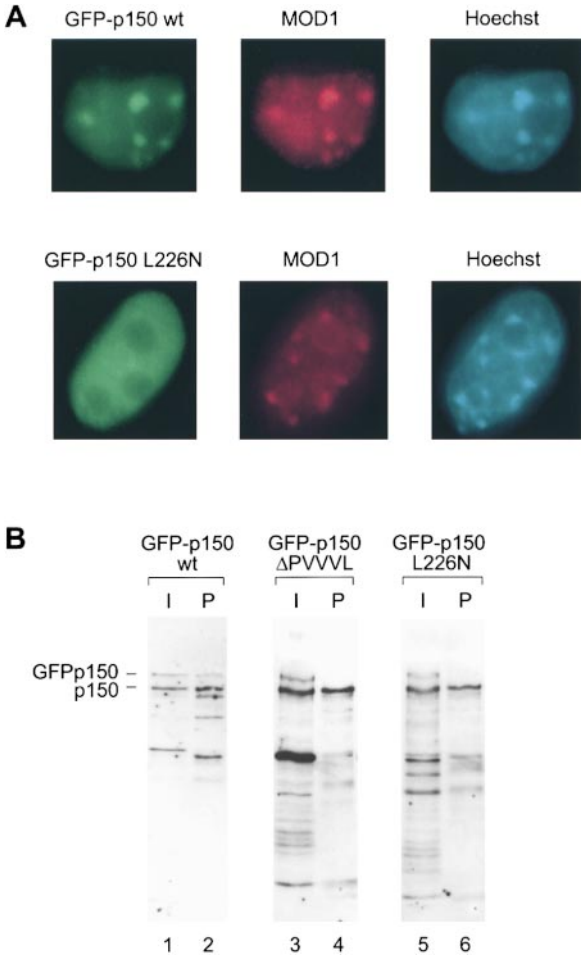

Figure 5. MIR Mutations Prevent Binding of CAF-1 p150 to HP1 Proteins in Heterochromatin In Vivo

Mouse L cells were transiently transfected with constructs expressing either wild-type GFP–p150 or the p150 MIR mutants ΔPVVVL or L226N.

(A) Targeting of the GFP fusion proteins to heterochromatin was examined 16 hr after transfection by immunofluorescence with antibodies against endogenous MOD1 (red) or staining DNA with Hoechst 33258 (cyan).

(B) A nuclear extract was prepared 24 hr after transfection and incubated with HP1α monoclonal antibody beads. The amounts of endogenous p150 and transiently expressed GFP–p150 that remained bound to beads following extensive washes were determined by Western blotting with a rabbit polyclonal antibody that detects both endogenous p150 and transfected GFP–p150. Lanes labeled I show the equivalent of 4% of input protein. The positions where endogenous p150 and transiently expressed GFP–p150 migrate are shown.

initially directed to euchromatic DNA replication foci and, following completion of euchromatin replication, CAF-1 is then recruited to heterochromatic replication foci (Krude, 1995). To determine whether binding to HP1 proteins was necessary for this recruitment of CAF-1 to DNA replication foci during S phase, wild-type and MIR mutants of p150 were expressed as GFP fusion proteins and their targeting to DNA replication foci was determined with antibodies against PCNA. As shown in Figure 6, MIR mutations did not affect the recruitment of p150 to either euchromatic or heterochromatic DNA replication foci. However, in cells that were not in S phase and did not stain with PCNA, point mutations of conserved MIR residues prevented the accumulation of p150 in heterochromatin. Therefore, although the MIR was dispensable for recruitment of p150 to euchromatic or heterochromatic DNA replication foci, it was necessary to keep p150 associated with HP1 proteins in heterochromatin outside of S phase.

Dissociation of HP1 and p150 from Heterochromatin in Late G2 and M Phase Occurs in Parallel with Histone H3 Phosphorylation

Experiments designed to determine the fate of GFP–p150 in mitotic cells failed to detect this protein during mitosis. This was not due to a cell cycle arrest induced as a result of overexpression of GFP–p150, because this protein was also undetectable in stably transfected mitotic cells (data not shown), where the level of expression of GFP–p150 was much lower. To determine the fate of GFP–p150 and HP1 proteins between G2 and M phase, we used a recently characterized antibody against phosphorylated histone H3 to detect G2 cells in an asynchronous population. Global histone H3 phosphorylation begins in pericentric heterochromatin in G2 cells and subsequently progresses to cover the entire genome prior to the onset of mitosis (Hendzel et al., 1997).

Figures 7A–7D show a series of immunofluorescence images obtained with a monoclonal antibody against MOD1. The temporal order of these images was determined based upon the known progression of H3 phosphorylation during G2 and M phase (Hendzel et al., 1997). Unexpectedly, we found that not only MOD1, but the three mouse HP1 family members (MOD1, MOD2, and HP1α) underwent large-scale, but progressive, dissociation from heterochromatin in G2 cells that was temporally linked to H3 phosphorylation (Figures 7A–7D and data not shown). We also observed this dissociation upon transient expression of GFP–MOD1 (data not shown), indicating that the monoclonal antibody we used to detect endogenous MOD1 was indeed specific for MOD1. This gradual dissociation was most obvious when cells that were extracted with the nonionic detergent Triton X-100 prior to formaldehyde fixation were compared with cells that were not extracted. The Triton extraction procedure removed the fraction of HP1 proteins that had already dissociated from heterochromatin, while leaving behind the HP1 proteins that remained bound to heterochromatin. For instance, in G2 cells where H3 phosphorylation was limited to pericentric heterochromatin and dissociation of HP1 proteins had just begun, a considerable amount of HP1 remained in heterochromatin whether the cells were extracted with detergent or not (Figure 7A, arrow). At this stage in G2, a considerable amount of GFP–p150 was still associated with heterochromatin although, as for MOD1, some of it was also diffuse throughout the nucleus (Figure 7E). In contrast, later in G2 when H3 phosphorylation covered the entire genome, most of the HP1 proteins had dissociated from heterochromatin and were readily extracted from the cells with Triton (compare Figures 7A and 7B, arrows). In both late G2 and M phase cells, GFP–p150

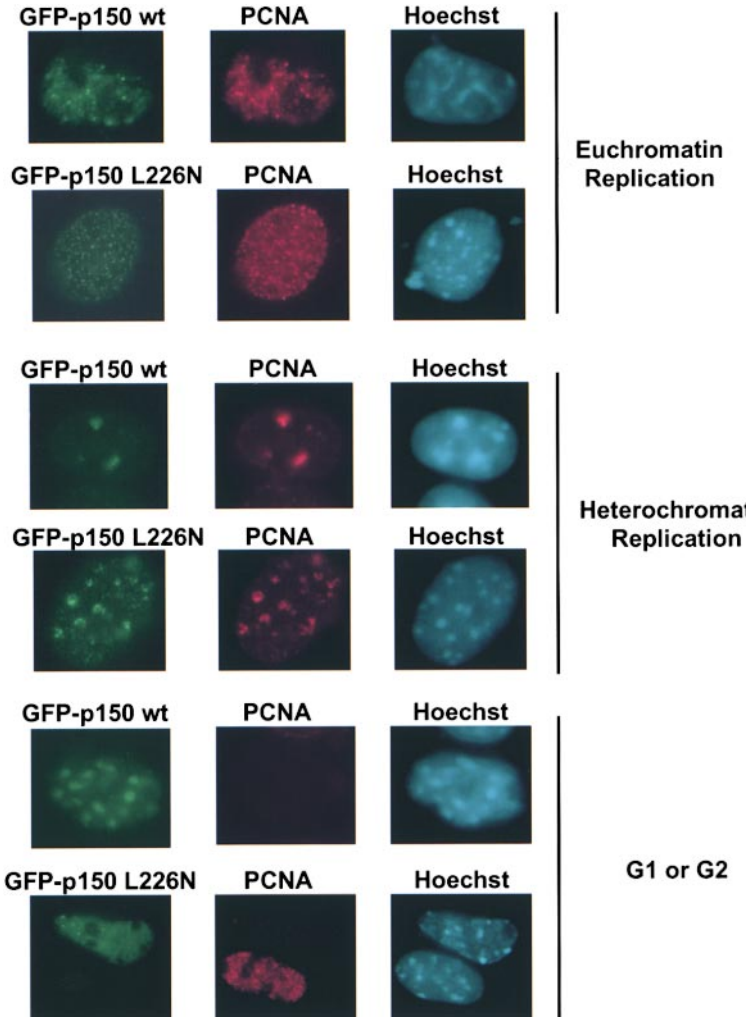

Figure 6. MIR Mutations Do Not Affect the Recruitment of CAF-1 p150 to Either Euchromatic or Heterochromatic DNA Replication Foci during S Phase but Prevent Its Association with Heterochromatin in Non–S Phase Cells

MIR mutations do not affect the recruitment of CAF-1 p150 to either euchromatic or heterochromatic DNA replication foci during S phase but prevent its association with heterochromatin in non–S phase cells mouse L cells were transiently transfected with constructs expressing either wild-type GFP–p150 or the MIR mutant GFP–p150 L226N. The cells were extracted with Triton 14 hr following transfection, prior to formaldehyde fixation and immunofluorescence microscopy. PCNA antibodies (red) were used to detect DNA replication foci (red) and limiting amounts of Hoechst 33258 to detect heterochromatin (cyan). The PCNA-positive cell in the GFP–p150 L226N panel was not transfected and serves as a control to illustrate the difference between an S phase cell and the upper non–S phase cell.

was undetectable (data not shown). GFP–p150 most likely dissociated from heterochromatin along with the HP1 proteins in late G2 and M phase and either became too diffuse to be detectable and/or was degraded. At least some degradation of p150 is likely to occur in vivo because the p150 subunit of CAF-1 contains PEST motifs and fragments of p150 can be detected by Western blotting (Figure 5B). Dissociation of HP1 proteins was also obvious in mitotic cells, at least until late anaphase/telophase. In mitotic cells that were not extracted with Triton, due to the absence of nuclear envelope, the large amount of HP1 proteins that were dissociated from heterochromatin gave rise to a strong, but diffuse signal throughout the entire cell volume (Figure 7C). Figure 7D shows two cells in late mitosis that were extracted with Triton before formaldehyde fixation. The cell labeled with the white arrow is in anaphase and still exhibits strong phosphorylation of histone H3. In this cell, most of the MOD1 protein is extracted by Triton (compare Figures 7C and 7D, white arrow), although some MOD1 also remained as a diffuse signal and a small fraction even colocalized with DNA stained with Hoechst. In contrast, the cell labeled with the yellow arrow in Figure 7D is in late anaphase/telophase, and histone H3 has been almost completely dephosphorylated. In this cell, a significant amount of MOD1 was Triton resistant and colocalized with mitotic chromosomes stained with Hoechst. Therefore, in late anaphase/telophase, MOD1 reassociated with mitotic chromosomes in parallel with histone H3 dephosphorylation.

Discussion

This paper describes the identification of a short MIR that is necessary for CAF-1 p150 to remain stably associated with mouse HP1 proteins in the heterochromatin of cells that are not in S phase. Because the MIR is located within the N-terminal domain of p150, a 300–amino acid region that is completely dispensable for nucleosome assembly during SV40 DNA replication in vitro, we propose that the function of the MIR is distinct from the role of CAF-1 in nucleosome assembly during S phase. Consistent with this, we find that p150 MIR mutations do not affect the recruitment of CAF-1 to either euchromatic or heterochromatic DNA replication foci during S phase but prevent its association with HP1 in heterochromatin outside of S phase (Figures 5 and 6). During S phase, the recruitment of CAF-1 to sites

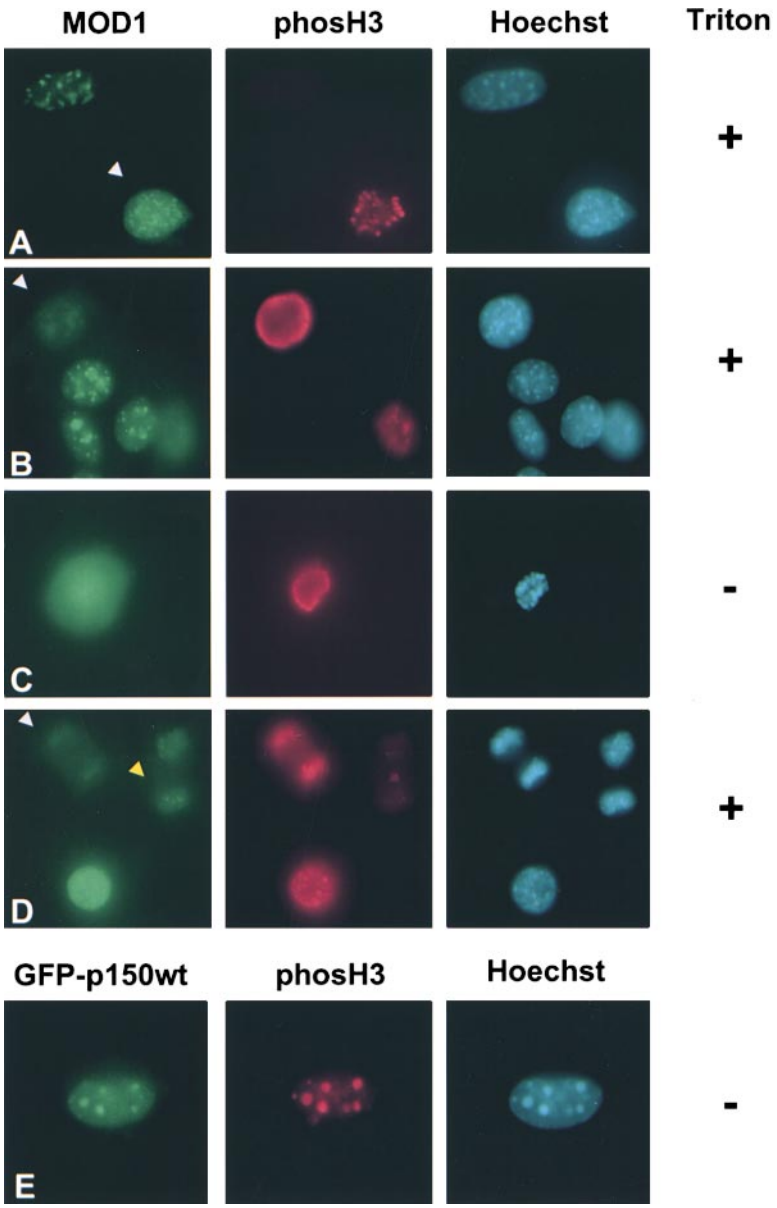

Figure 7. MOD1 and GFP–p150 Progressively Dissociate from Heterochromatin prior to the Onset of Mitosis in Parallel with Histone H3 Phosphorylation, and MOD1 Rebinds to Chromatin in Late Anaphase or Telophase

Mouse NIH 3T3 cells were either fixed directly with formaldehyde (C and E) or extracted with Triton prior to formaldehyde fixation (A, B, and D) and processed for double immunofluorescence with antibodies against MOD1 (green) and phosphorylated histone H3 (red). DNA was stained with Hoechst 33258 (cyan). The images in (A)–(E) are arranged according to the known progression of H3 phosphorylation from G2 to the end of mitosis (Hendzel et al., 1997). (E) Mouse NIH 3T3 cells transfected with a construct for expression of wild-type GFP–p150 were stained with antibodies specific for phosphorylated H3. Arrows indicate the cells referred to in the text. In (D), the cell indicated by the white arrow is in anaphase, while the cell shown by the yellow arrow is either a very late anaphase or a telophase cell.

of DNA synthesis is mediated, at least in part, by an interaction with PCNA (Shibahara and Stillman, 1999), a processivity factor for DNA polymerase δ that is required for both euchromatic and heterochromatic DNA replication.

Heterochromatin Maintenance in Proliferating Cells

The maintenance of heterochromatin requires the synthesis of both new histones and heterochromatin proteins and their orderly assembly during DNA replication. Very little is known about how newly synthesized heterochromatin proteins that do not bind to DNA directly, such as HP1, are preferentially targeted and retained into heterochromatin. Heterochromatin replication is an attractive period of the cell cycle where this could occur. Since CAF-1 is almost exclusively found in heterochromatin during its replication in late S phase (Figure 6), CAF-1 could bring both newly synthesized histones and HP1 proteins to replicating heterochromatin. Indeed, we showed that CAF-1 p150 and MOD1 remain bound to each other in the nascent chromatin formed in a cell-free system for nucleosome assembly during DNA replication (Figure 3). In vivo however, GFP–MOD1 expressed during euchromatic DNA replication is targeted specifically to heterochromatin (Figure 4). During early S phase, the bulk of the CAF-1 protein is present in euchromatic DNA replication foci (Figure 6). Therefore, targeting of MOD1 to heterochromatin in early S phase is most likely CAF-1 independent. This result also shows that targeting of new MOD1 molecules to heterochromatin does not require that their synthesis be strictly coupled to heterochromatin replication in late S phase. In this respect, there is an interesting contrast between MOD1 and the pericentric heterochromatin histone CENP-A. CENP-A is a histone H3 variant whose expression is normally restricted to late S phase. Unlike MOD1,

CENP-A is misincorporated into euchromatin when expressed early in S phase from a histone H3 promoter (Shelby et al., 1997). In contrast to CENP-A, HP1 proteins do not bind to DNA directly. The simplest model consistent with all the available evidence is that newly synthesized HP1 proteins are specifically targeted to heterochromatin because they can recognize a preexisting component of heterochromatin other than DNA.

The biological rationale behind the p150-HP1 interaction may be analogous to that proposed for the interaction between the ORC and HP1. ORC provides anchoring sites for HP1 proteins in *Drosophila* pericentric heterochromatin, by virtue of its ability to bind directly to DNA (Huang et al., 1998). Unlike ORC, CAF-1 p150 molecules that remain part of heterochromatin following replication may stabilize the association of HP1 proteins with heterochromatin indirectly by binding to histones. Alternatively, an interaction between CAF-1 and either preexisting or new MOD1 molecules may be necessary only transiently during heterochromatin replication, in order to reestablish heterochromatin structure following its disruption during DNA replication. Clearly, further studies are necessary to determine the precise function of the interaction between CAF-1 and HP1 proteins.

CAF-1 is required for the maintenance of telomeric silencing in *S. cerevisiae*, but this yeast does not contain an obvious homolog of HP1. We suggest, however, that the silent information regulator (SIR) proteins perform a similar function and that yeast CAF-1 should bind to one or more of the SIR proteins in yeast heterochromatin, thereby performing a similar function to the CAF-1-HP1 interaction.

Mouse HP1 Proteins Dissociate from Heterochromatin from G2 to Late M Phase

Pericentric heterochromatin is generally regarded as constitutive (i.e., it remains visibly condensed even during interphase). Although some dissociation of HP1 from mitotic chromosomes has been reported in *Drosophila* (Kellum et al., 1995), the finding that mouse heterochromatin undergoes drastic changes in structure that occur in parallel with H3 phosphorylation prior to the onset of mitosis was unexpected. The large-scale dissociation of mouse HP1 proteins from heterochromatin reported here seems at first glance paradoxical, given that null alleles of HP1 in *Drosophila* and *swi6* in *S. pombe* result in a number of structural defects in mitotic chromosomes (Ekwall et al., 1995; Kellum and Alberts, 1995). This argues that HP1 proteins are required for proper mitotic chromosome segregation. The answer to this paradox probably lies in the fact that, in spite of the large-scale dissociation of HP1 in late G2, a fraction of HP1 molecules remain bound to pericentric heterochromatin throughout mitosis (Wreggett et al., 1994; Remboutsika et al., 1999; Yamada et al., 1999). The dissociation of HP1 proteins prior to mitosis must therefore be regulated in such a way that the fraction of HP1 molecules required for proper mitotic chromosome segregation remains bound to pericentric heterochromatin.

Intriguingly, the progressive dissociation of the CAF-1 p150 and HP1 proteins in G2 occurs in parallel with H3 phosphorylation, which initiates in pericentric heterochromatin and progressively spreads into euchromatin until the whole genome contains phosphorylated H3 (Hendzel et al., 1997). During late anaphase/telophase, H3 is dephosphorylated (Hendzel et al., 1997) and the HP1 proteins rebind to mitotic chromosomes as they progressively decondense. We suggest that the function of this dramatic dissociation of HP1 from heterochromatin is to allow access to various proteins that promote faithful mitotic chromosome segregation. For instance, such proteins might include the H3 kinase and/or proteins such as topoisomerase II or the condensins (Hirano, 1999). CAF-1 p150 remains associated with heterochromatin at least until the early stages of H3 phosphorylation in G2. In late G2 and mitotic cells, CAF-1 p150 is virtually undetectable by immunofluorescence (this paper; Krude, 1995). Marheineke and Krude (1998) recently reported that CAF-1 p60 is hyperphosphorylated in nocodazole-arrested human cells. This mitotic form of CAF-1 is not bound to mitotic chromosomes and is inactive in nucleosome assembly. It seems therefore likely that the CAF-1 protein is phosphorylated and inactivated in late G2 and mitotic cells. The precise relationship between this cell cycle–regulated inactivation of CAF-1 and the dynamic changes in heterochromatin that occur in late G2 and mitotic cells remains to be determined. Clearly, the heterochromatin of higher eukaryotes cannot be regarded as a static structure during the course of the cell cycle. Further work is needed to assess the function of these dynamic changes in heterochromatin and how these events are regulated by the cell cycle machinery.

Experimental Procedures

DNA Manipulations

For the two-hybrid screen, the *S. cerevisiae* reporter strain L40 (*MAT***a** *trp1 leu2 his3 LYS2::lexA-HIS3 URA3::lexA-lacZ*) was transformed with a plasmid expressing either full-length MOD1 or the shadow chromodomain of MOD1 fused to the LexA DNA-binding domain and used as baits to screen a mouse embryo cDNA library fused to the VP16 activation domain. Clones encoding MOD1-interacting proteins were scored for both *lacZ* expression and histidine prototrophy. Positive clones whose interaction was specific for MOD1 were sequenced and used to isolate a full-length cDNA for p150 using a combination of RACE PCR from a mouse liver cDNA library (Clontech) and screening of a mouse embryo phagemid library in the λExlox vector (Novagen). A full-length mouse p150 cDNA was cloned into the BamHI and NotI sites of the pCITE4a(+) vector (Novagen) to create plasmid pCITE4a mp150wt for in vitro translation in rabbit reticulocyte lysate or into the BglII and Bsp120I sites of the EGFP-C1 vector (Clontech) for transient expression of p150 as a GFP fusion protein in mammalian cells (plasmid C1 mp150wt). The MIR mutations in p150 were made by site-directed mutagenesis of the pCITE4a mp150wt construct using QuickChange (Stratagene) and subcloned into the EGFP-C1 vector to create plasmids C1 mp150 V224S, V224D, L226N, and ΔPVVVL. A cDNA fragment for expression of GST–p150 MIR (residues 176–327) was subcloned into the BamHI and EcoRI sites of the pGEX-5X vector (Pharmacia) to create pGEX5X p150 MIR. Constructs for expression of GFP–MOD1 from either a CMV promoter or the mouse H3 promoter were made by cloning the MOD1 cDNA into the HindIII and BamHI sites of the EGFP-C1 vector to create C1 mMOD1 and by subcloning the GFP–MOD1 fragment into the NcoI and MluI sites of the pMH3.2–614 vector (Taylor et al., 1986) to create pMH3.2 GFPmMOD1. The constructs generated by PCR amplification of cDNA fragments were verified by sequencing.

Immunofluorescence

Similar results were obtained with either mouse NIH 3T3 or L cells grown in DMEM plus 10% calf serum on poly-L-lysine-coated glass

coverslips. Transfection of 1×10^5 cells in a 3 cm dish was performed with 4 μg plasmid DNA and 10 μl Superfect reagent (Qiagen) for 2 hr at 37°C. Following removal of the transfection reagent, the cells were incubated at 37°C for 12 hr prior to immunofluorescence. Cells were either fixed immediately with 2% paraformaldehyde in phosphate-buffered saline (PBS) or extracted on ice for 5 min with 0.2% Triton X-100 in PBS plus 1% goat serum prior to paraformaldehyde fixation. For PCNA detection, the cells were always Triton-extracted prior to fixation and treated by addition of cold methanol (−20°C) for 10 min at room temperature prior to incubation with the PC10 monoclonal antibody (Waseem and Lane, 1990). This antibody did not detect PCNA when methanol treatment was omitted. MOD1, MOD2, and HP1α were detected with mouse monoclonal antibodies specific for each protein (Remboutsika et al., 1999). Phosphorylated histone H3 was detected using a rabbit polyclonal antibody (Upstate Biotechnology). The secondary antibodies used were either anti-mouse or anti-rabbit IgG coupled to fluorescein or Texas red (ICN). The location of heterochromatin was monitored by staining DNA with 50 ng/ml Hoechst 33258. Under these conditions, the dye preferentially intercalates into the large amounts of AT-rich DNA present in the pericentric heterochromatin of mouse cells. Bleed-through between the green fluorescent protein, Hoechst, and Texas red signals was negligible.

Chromatin Assembly during SV40 DNA Replication In Vitro

These assays were performed as described previously (Kaufman et al., 1995; Verreault et al., 1996). Following DNA replication for 1 hr at 37°C, antibodies bound to protein G-Sepharose were added for 2 hr at room temperature. The beads were washed (25 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 10% glycerol, and 0.01% NP40), resuspended (50 mM Tris-HCl [pH 8.0], 10 mM EDTA, and 0.2% SDS), and treated with 1 mg/ml pronase at 37°C for 1 hr. Immunoprecipitated DNA was extracted with phenol-chloroform, precipitated with ethanol, and analyzed by agarose gel electrophoresis.

Binding of CAF-1 Subunits to His–MOD1

Wild-type human p150 or p150 lacking amino acids 1–296 were produced by in vitro translation in the rabbit reticulocyte lysate. Recombinant CAF-1 containing the three subunits of the protein or CAF-1 lacking the p150 subunit were expressed and purified as described previously (Kaufman et al., 1995; Verreault et al., 1996). Recombinant His-tagged MOD1 or the MOD1 chromo- and shadow chromodomains were expressed and purified from *E. coli* (Ball et al., 1997). Nickel-NTA-agarose beads with or without His–MOD1 were used in binding assays in buffer AI300 (10 mM Tris-HCl [pH 7.7], 50 mM imidazole, 300 mM NaCl, 10% glycerol, 0.02% Nonidet-P40, 1 mM 2-mercaptoethanol). After extensive washes in buffer AI300, the proteins that remained bound to nickel-NTA-agarose beads were detected by SDS-polyacrylamide gel electrophoresis and either fluorography or Western blotting.

Binding of MOD1 to GST–MIR

The MOD1, MOD1 chromodomain, and MOD1 shadow chromodomain proteins purified from *E. coli* were incubated in buffer A200 (20 mM Tris-HCl [pH 7.5], 200 mM NaCl, 10% glycerol, 0.05% Nonidet-P40, and 1 mM DTT) with the purified GST–MIR fusion protein (residues 176–327 of mouse p150) bound to glutathione agarose beads. After extensive washes in buffer A200, the proteins that remained bound to GST–MIR were detected by electrophoresis through a tricine 16% polyacrylamide gel and Coomassie blue staining.

Coimmunoprecipitation of p150 and HP1α from Nuclear Extracts

Nuclear extracts from mouse L cells transfected with constructs expressing either wild-type or MIR mutants of p150 were prepared in buffer B400 (25 mM Tris-HCl [pH 7.2], 400 mM NaCl, 10% glycerol, 0.02% Nonidet-P40, and 1 mM DTT). This extract was supplemented with 100 μg/ml ethidium bromide and incubated for 1 hr at 4°C with HP1α antibody beads. The beads were washed extensively with buffer B400 plus ethidium bromide before detection of coimmunoprecipitated CAF-1 p150 by Western blotting. This procedure only extracted a small fraction of HP1α from heterochromatin. Results similar to those shown in Figure 5B were also obtained by extracting part of the remaining HP1α with buffer B1000 (buffer B plus 1 M NaCl, diluted to B400 plus ethidium bromide prior to immunoprecipitation).

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Note Added in Proof

Two recent papers address issues that are related to the data presented in this report. Ryan et al. report an interaction between the KAP-1 (TIF1β) corepressor and both mouse and human HP1 proteins dependent upon amino acid sequences related to the MIR identified in this report. In a second paper, Minc et al. (1999) report on the dynamic nature of mouse HP1 proteins during mitosis.

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