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The p150 and p60 Subunits of Chromatin Assembly Factor I: A Molecular Link between Newly Synthesized Histones and DNA Replication

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Summary

Chromatin assembly factor I (CAF-I) from human cell nuclei is a three-subunit protein complex that assembles histone octamers onto replicating DNA in a cell-free system. Sequences of cDNAs encoding the two largest CAF-I subunits reveal that the p150 protein contains large clusters of charged residues, whereas p60 contains WD repeats. p150 and p60 directly interact and are both required for DNA replication-dependent assembly of nucleosomes. Deletion of the p60-binding domain from the p150 protein prevents chromatin assembly. p150 and p60 form complexes with newly synthesized histones H3 and acetylated H4 in human cell extracts, suggesting that such complexes are intermediates between histone synthesis and assembly onto replicating DNA.

Introduction

The DNA of eukaryotic organisms is packaged in the nucleus with an approximately equal mass of protein, forming a complex nucleoprotein structure known as chromatin. The most fundamental repeat structure of chromatin is the nucleosome core particle, comprised of 146 bp of DNA wrapped around an octamer of histone proteins (two molecules of each of the four core histones, H2A, H2B, H3, and H4). Packaging of DNA into nucleosomal structures is generally inhibitory to processes requiring access to the DNA, including transcription (reviewed by Felsenfeld, 1992; Paranjape et al., 1994). Conversely, activation of genes is often correlated with an increased nuclease sensitivity within regulatory regions, indicative of an altered chromatin structure. Propagation of such so-called open chromatin regions over the course of multiple cell divisions is an important aspect of maintaining cellular differentiation. Therefore, insights into mechanisms involved in the assembly and inheritance of chromatin structures are an important goal for the study of eukaryotic gene regulation.

Assembly of new nucleosomes is closely linked temporally to the passage of the replication fork in somatic cells. After replication, nascent DNA displays transient sensitivity to nucleases, and only after 10–20 min does newly synthesized chromatin display a digestion pattern and nucleosomal spacing similar to that of unreplicated chromatin (e.g., Levy and Jakob, 1978; Smith et al., 1984). Also, histones H3 and H4 are assembled onto nascent DNA almost immediately after replication-fork passage, whereas histones H2A and H2B are added to DNA after 2–10 min (Worcel et al., 1978). This same order of addition has been observed in completely defined systems in which purified histones and DNA are mixed in 2 M NaCl and assembled into nucleosomes by dialysis (Hansen et al., 1991). The universality of this pathway probably reflects the fact that H3 and H4 are the most tightly bound and centrally located histones of the core particle (see van Holde, 1989; Wolffe, 1992).

Extracts of egg or embryonic cells of Xenopus and Drosophila promote assembly of regularly spaced nucleosomes (Laskey et al., 1977; Glikin et al., 1984; Becker and Wu, 1992; Kamakaka et al., 1993). Xenopus oocytes contain large amounts of nucleoplasmin bound to histones H2A and H2B and N1/N2 bound to histones H3 and H4 (Kleinschmidt et al., 1985; Dilworth et al., 1987) Nucleoplasmin and N1/N2 are acidic proteins that sequester the large maternal store of histones and assemble large amounts of chromatin during the rapid early embryonic cell divisions. However, these factors do not require DNA replication to assemble nucleosomes.

In contrast with embryonic cells, somatic cells do not store large pools of histones H3 and H4, but instead synthesize them in a largely cell-cycle dependent manner at the onset of DNA synthesis (Wu and Bonner, 1981). A three-subunit protein 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). CAF-I differs from other assembly factors because it does not promote significant nucleosome assembly in the absence of ongoing DNA replication. 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, 1991a). 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.

CAF-I is also unique among nucleosome assembly factors because it assembles specific histones onto DNA. CAF-I cannot assemble histones H3 and H4 purified from cellular chromatin onto DNA. Instead, CAF-I uses the histones H3 and H4 present in cytosolic extracts of human cells (Smith and Stillman, 1989, 1991a). These histones are newly synthesized, as detected by pulse labeling (Smith and Stillman, 1989, 1991a; see below). In vivo, there is a burst of histone H3 and H4 synthesis at the onset of S phase (Wu and Bonner, 1981), and the newly synthesized histone H4 is transiently acetylated on lysine residues near the N-termini while still in the cytoplasm (Ruiz-Carillo et al., 1975; Jackson et al., 1976; Sobel et al., 1994, 1995). This modification is lost during entry into

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the nucleus and assembly into chromatin. Also, acetylated histone H4 in cytosolic extracts is in a complex that contains histone H3 (Perry et al., 1993). When deacetylase enzymes are inhibited, nucleosomes assembled onto newly replicated DNA are enriched in acetylated H4 (Perry et al., 1993). Thus, mechanisms exist to target newly synthesized histones to the DNA replication fork.

We present here a molecular characterization of replication-linked chromatin assembly proteins, the p150 and p60 subunits of the human CAF-I complex. Newly synthesized histones H3 and H4 in cell extracts form complexes with p150 and p60, suggesting that the CAF-I proteins in these complexes are involved in escorting newly synthesized histones to sites of DNA replication–linked chromatin assembly.

Results

Molecular Cloning of p150 and p60 cDNAs

We cloned a cDNA encoding the p150 subunit of CAF-I using a combination of antibody screening and protein sequencing (Figure 1; see Experimental Procedures). Except for partial human cDNA sequences, there are currently no close homologs of the p150 gene in sequence data bases. However, the p150 gene has several striking features. The gene is predicted to encode a highly charged protein with an isoelectric point of 5.5. Furthermore, there are several regions with large clusters of acidic residues. The p150 gene also has a PEST box, which is defined as a region enriched in proline (P), glutamic acid (E), serine (S), threonine (T) and also aspartic acid residues (D) (Rogers et al., 1986). The presence of these motifs in proteins has been correlated with short in vivo half-lives. Indeed, the p150 protein undergoes proteolysis in cellular extracts (see below).

A cDNA encoding the p60 subunit of CAF-I was cloned using amino acid sequence data (Figure 2A). p60 is a member of the tryptophan–aspartate (WD), or transducin, repeat family of proteins (reviewed by Neer et al., 1994). Such proteins contain four to eight repeated motifs; each motif is approximately 40 amino acids in length, often ending in WD residues (Figure 2B). p60 itself has seven repeats, although the fifth and sixth repeats show significant divergences from canonical residues. The structural or biological significance of these repeats is presently ambiguous, since they have been found in many eukaryotic organisms and in a large number of proteins with greatly different functions and cellular localizations. However, a

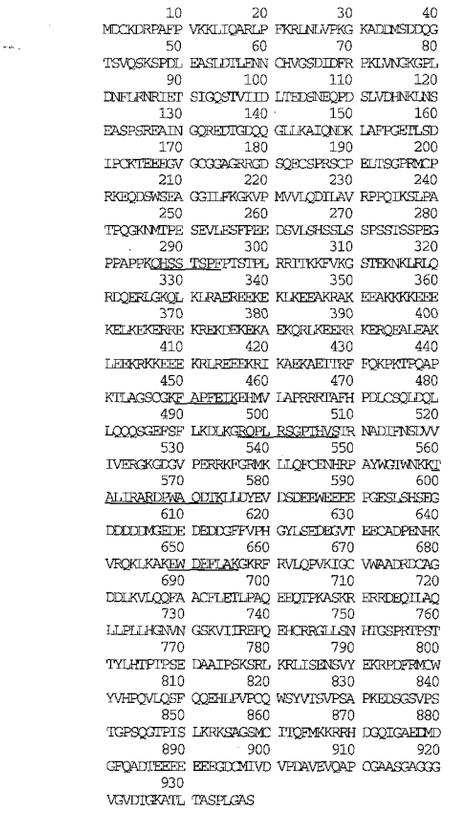

Figure 1. Predicted Amino Acid Sequence of the CAF-I p150 Subunit

Peptide sequences obtained from the p150 subunit of the CAF-I complex are underlined.

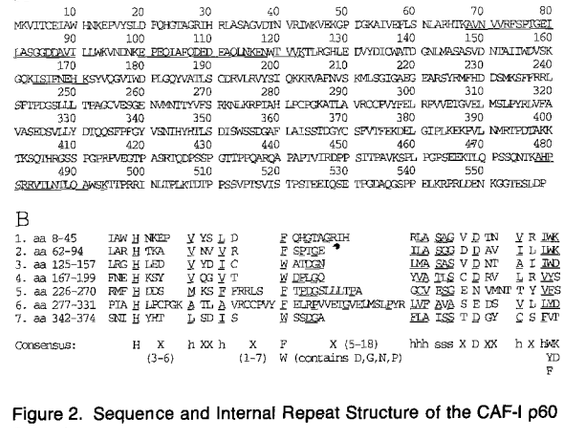

Figure 2. Sequence and Internal Repeat Structure of the CAF-I p60 Subunit

(A) Predicted amino acid sequence. Peptide sequences obtained from the p60 subunit of the CAF-I complex are underlined. Amino acids 100–116 and 117–123 were sequenced as different peptides. The tryptophan at amino acid 119 and the tryptophan and serine at amino acids 492–493 could not be unambiguously determined from the protein sequence data.

(B) WD repeat structure of p60. Numbers of amino acids (aa) are indicated next to the primary sequence of each of the seven repeats. Most nonconserved amino acids between repeats are omitted for brevity. Underlined residues conform to the consensus shown at the bottom. This alignment was made by hand; the derived consensus for the repeats in the p60 is very similar to that found previously by examination of a large number of WD protein sequences, although some differences are observed (e.g., Neer et al., 1994). h represents any of the hydrophobic amino acids L, I, V, M, F, C, or A; s represents the small amino acids S, A, G, or T; x indicates that any amino acid can be found at that position.

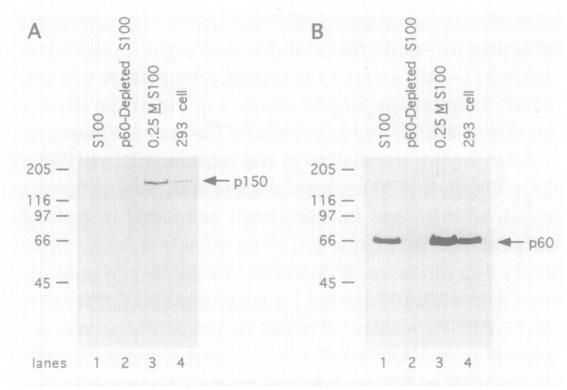

Figure 3. Immunological Characterization of Human Cell Extracts

Samples were separated on a 10% SDS–polyacrylamide gel and immunoblotted. Lane 1, ~65 μg of 293 cell S100 cytosolic extract; lane 2, ~54 μg of anti-p60 MAb 24–depleted S100 extract; lane 3, ~75 μg of S100 made in the presence of 0.25 M NaCl; lane 4, total 293 cell proteins. Approximately 10^7 cells were lysed in 1 ml of SDS sample buffer; 5 μl was analyzed on this gel.

(A) Detection of p150. The filter was probed with anti-p150 MAbs 1 and 48.

(B) Detection of p60. The same filter shown in (A) was stripped and reprobed with anti-p60 MAbs 96 and 53.

recent study has shown that a single WD repeat can act as a direct protein–protein interaction domain (Komachi et al., 1994). The C-terminus of the p60 gene also contains a PEST region.

Chromatin Assembly by Recombinant p150 and p60

Initial experiments showed that the p150 protein was sufficient to promote DNA replication–linked chromatin assembly in the presence of S100 extract, a cytosol extract made by disrupting human 293 cells at low ionic strength (see Figure 4B, lanes 14–17; data not shown). However, the S100 extract contained substantial amounts of the p60 subunit (Figure 3B, lane 1). In contrast, the p150 subunit was not abundant in the S100 cytosol extract, although it could be easily detected in whole-cell lysates or in an S100 extract made by disrupting cells in the presence of 0.25 M NaCl, which contains more nuclear proteins (Figure 3A, lanes 1, 3, and 4). Therefore, to test whether p60 was essential for the chromatin assembly reaction, we depleted the p60 from the S100 extract by immunoaffinity chromatography with an anti-p60 monoclonal antibody (MAb) resin (Figure 3B, lane 2; Figure 4B).

Recombinant baculoviruses containing either the p150 or p60 genes were generated, and the p150 and p60 proteins were purified from extracts of infected insect cells (Figure 4A). The purified proteins were added to DNA replication reactions to test for assembly of nucleosomes (Stillman, 1986; Smith and Stillman, 1989). In this assay, DNA replication of plasmids bearing the SV40 origin is dependent on SV40 T antigen and a human cell cytosolic extract (S100) that contains the necessary DNA polymerases and auxiliary factors. Addition of purified CAF-I to this reaction promotes the formation of nucleosomes dur-

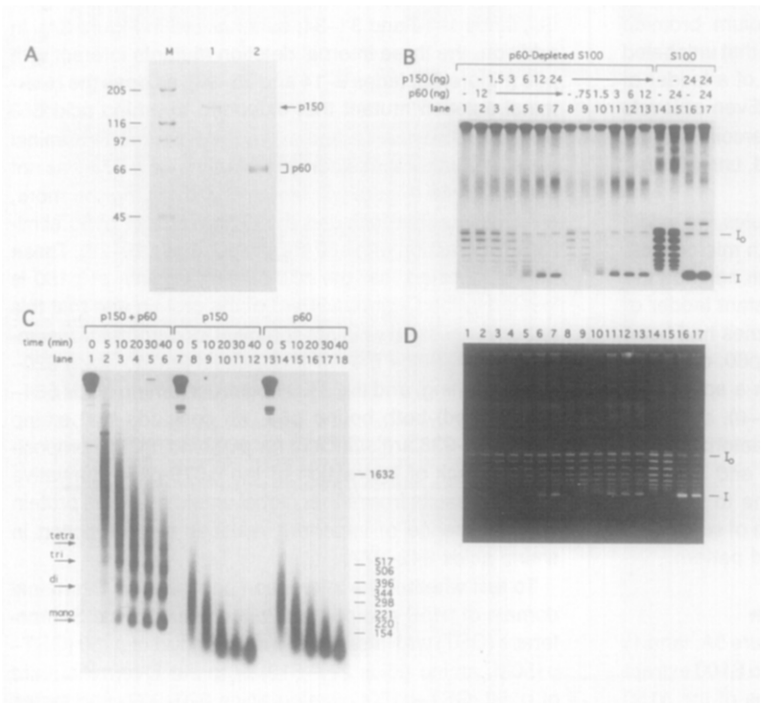

Figure 4. Purification and Characterization of Recombinant p150 and p60 Proteins

(A) Approximately 0.75 μg of p150 (lane 1) and 1.5 μg of p60 (lane 2) Q-Sepharose fractions purified from insect cells were analyzed on a 10% SDS–polyacrylamide gel and stained with Coomassie blue. M indicates molecular mass markers, with mass in kilodaltons.

(B) Chromatin assembly by recombinant p150 and p60. SV40 replication reactions (50 μl) were performed with anti-p60 MAb 24–depleted cytosolic S100 extract (lanes 1–13) or with undepleted S100 (lanes 14–17). Extracts were supplemented with the indicated amounts of purified recombinant p150 or p60 or both. Replication products were analyzed on a 1% agarose gel and autoradiographed. Migration of form I negatively supercoiled and form I_0 relaxed monomer circle DNA is indicated.

(C) Micrococcal nuclease digestion of assembled chromatin. Replication reactions (300 μl) were supplemented with 144 ng of insect cell p150 plus 72 ng of p60 (lanes 1–6), 144 ng of p150 alone (lanes 7–12), or 72 ng of p60 alone (lanes 13–18). Aliquots of replication products (50 μl) were digested for the indicated amounts of time. Migration of linear double-strand DNA markers is indicated along the right, with lengths in base pairs. The migration of mononucleosome-, disome-, trisome- and tetrasome-length material is indicated by the arrows.

(D) Replication-preferential assembly by p150 and p60. Total DNA in the gel shown in (B) was visualized by ethidium bromide staining. Note the presence of an unradiolabeled ladder of topoisomers in reactions that underwent efficient assembly of replicated molecules (e.g., lanes 6, 7, and 11–13).

ing DNA replication. The degree of nucleosome assembly is assessed by the superhelical state of radiolabeled DNA after removal of proteins. Since each nucleosome constrains one negative supercoil (Simpson et al., 1985), efficient nucleosome formation is indicated by the migration of replicated DNA as a highly negatively supercoiled form (termed form I) in native agarose gels.

Addition of both p150 and p60 was required for chromatin assembly in the p60-depleted extract, but only p150 was required in the undepleted extract (Figure 4B), consistent with the immunoblotting results (see Figure 3). In the undepleted extract, 24 ng of p150 was sufficient to convert completely the replicated DNA in a standard 50 μl assay to a highly negatively supercoiled form (Figure 4B, lane 16; data not shown). In the p60-depleted extract, this amount of p150 would not form chromatin without addition of p60 (Figure 4B, lane 8); full supercoiling of the replicated DNA required 6–12 ng of p60 (~0.1–0.2 pmol) and 24 ng p150 (~0.2 pmol; Figure 4B). Approximately 50 ng (~0.2 pmol) of CAF-I purified from human cells is required for full assembly under these conditions (Smith and Stillman, 1989). Therefore, the specific activities of the recombinant p150 and p60 proteins were very similar to that of the CAF-I complex from human cells.

The recombinant p150 and p60 proteins assembled chromatin in a replication-preferential manner. Upon addition of high levels of these two recombinant proteins, nearly all of the radiolabled, closed circular monomer plasmid was negatively supercoiled (e.g., Figure 4B, lanes 6, 7, and 11–13). In contrast, analysis of total DNA recovered from these replication reactions by ethidium bromide staining of the same gel (Figure 4D) showed that unlabeled DNA present in these reactions consisted of a ladder of relaxed topoisomers present in all lanes. Even at levels of p150 and p60 that caused complete supercoiling of the replicated DNA, it is clear that the unlabeled, unreplicated DNA was not assembled into chromatin.

To better characterize the product molecules, we mildly digested chromatin assembly reactions with micrococcal nuclease. This cleaves linker DNA between neighboring nucleosomes, resulting in a nuclease-resistant ladder of bands indicating that an array of nucleosomes had been formed. In the presence of both p150 and p60, digestion of products yielded a ladder of bands with a spacing of approximately 190 bp (Figure 4C, lanes 2–6), similar to that previously described during the characterization of the CAF-I complex (Stillman, 1986; Smith and Stillman, 1989). Addition of either p150 or p60 alone to the p60-depleted S100 extract resulted in protection of some subnucleosomal-sized DNA without a repeated pattern.

Molecular Dissection of the p150 Protein

p150 synthesized by in vitro translation (Figure 5A, lane 1) supports chromatin assembly when added to S100 extract (Figure 5B, lanes 2 and 3). To map regions of the p150 protein required for activity, we constructed a series of N-terminal, C-terminal, and internal deletions of the p150 gene (diagrammed in Figure 6A). The three internal deletions created were: the PEST domain, amino acids 245–296; the highly charged KER domain, comprised chiefly of lysine (K), glutamic acid (E), and arginine (R), amino acids 311–445; and the ED region, amino acids 564–641, which includes the largest clusters of the acidic residues glutamic acid (E) and aspartic acid (D) in the p150 protein.

After in vitro translation of the mutated cDNAs (Figure 5A), roughly equimolar amounts of the various p150 derivatives were tested for chromatin assembly (Figure 5B; summarized in Figure 6A). The N-terminal 296 amino acids of p150 were dispensable for chromatin assembly activity in the S100 extract, because the Δ88, Δ204, Δ296, and ΔPEST proteins all acted as efficiently as wild-type p150 (Figure 5B, lanes 4–11). In contrast, internal deletion of the KER or ED regions destroyed detectable activity, as did any of the C-terminal deletions tested (Figure 5B, lanes 12–15 and 18–25). Also, a large N-terminal deletion to amino acid 678 yielded nonfunctional protein (Figure 5B, lanes 16 and 17).

We also tested in vitro translated p150 derivatives for binding to p60 using a coimmunoprecipitation assay. Wild-type and mutant p150 molecules were incubated with p60 and an anti-p60 MAb (Figure 5C, even-numbered lanes). As a negative control for nonspecific precipitation of p150, assays were also performed in the absence of p60 (Figure 5C, odd-numbered lanes). Coprecipitations were performed using purified recombinant p60 (Figure 5C, lanes 1–30) or with in vitro translated p60 (Figure 5C, lanes 31–38) with similar results. All of the p150 derivatives that are functional for chromatin assembly are precipitated by the anti-p60 antibody, but only in the presence of p60 (Figure 5C, lanes 1–10 and 31–34; summarized in Figure 6A). In addition, the three internal deletion mutants interact with p60 (Figure 5C, lanes 9–14 and 35–38), as does the N-terminal deletion mutant that extended to amino acid 619 (Figure 5C, lanes 15 and 16). However, the N-terminal deletion mutant that extended to amino acid 678 was not bound by p60 (Figure 5C, lanes 17 and 18). Furthermore, all deletions that included the C-terminus of p150 eliminated interaction with p60 (Figure 5C, lanes 19–28). These data suggested that the p60-binding domain of p150 is located in the C-terminal third of the protein and that this region is necessary but not sufficient for chromatin assembly activity. Since the Δ619 construct (amino acids 620–938 remaining) and the ΔED construct (amino acids 564–641 deleted) both bound p60, we conclude that amino acids 642–938 are sufficient for p60 binding. We propose that the lack of interaction of the Δ678 p150 derivative with p60 results from either global unfolding of this protein or the absence of important residues for interaction in amino acids 642–677.

To test whether the interaction between the C-terminal domain of p150 and p60 was direct, glutathione S-transferase (GST) was fused to the middle third of p150 (GST–p150M, amino acids 297–619) or to the C-terminal third of p150 (GST–p150C, amino acids 620–937) and tested for binding to in vitro translated p60 (Figure 6B, lanes 1–4) or purified recombinant p60 (Figure 6B, lanes 5–8). In both cases, unfused GST or GST–p150M did not bind p60, but GST–p150C did. This interaction was stable in the

presence of at least 0.5 M NaCl. The interaction between the p150 C-terminal third and p60 is therefore a direct protein–protein interaction.

Interactions among p150, p60, and Histones H3 and H4 in Cell Extracts

Newly synthesized human histone H4 is transiently acetylated on Lys-5 and Lys-12 (Sobel et al., 1995), and the histones H3 and H4 used by CAF-I for chromatin assembly are newly synthesized molecules in human cell cytosol extracts (Smith and Stillman, 1991a). We therefore tested whether the p150 or p60 CAF-I subunits interact with acetylated histone H4 present in cell extracts. For some experiments, extracts were prepared from human 293 cells that had been pulse labeled with ^{14}C-lysine and arginine to increase the sensitivity of detection of the newly synthesized histones. Immunoprecipitation experiments were performed using MAbs that recognize p150 and p60 (MAbs 1 and 24, respectively; Smith and Stillman, 1991b) and a rabbit antiserum raised against a synthetic peptide corresponding to the tetraacetylated Tetrahymena histone H4 N-terminus, which reacts with all acetylated forms of the protein (Lin et al., 1989). Also, to maintain the labile acetyl groups on the histones, sodium butyrate, an inhibitor of histone deacetylase enzymes, was added to the harvested cells prior to homogenization and at all subsequent steps of the experiments.

First, interactions between proteins present in the S100 cytosol extract were investigated. Immunoprecipitated proteins were analyzed by immunoblotting to detect p60. The anti-p60 MAb 24 did indeed precipitate the p60 protein in these experiments, but the control 12CA5 MAb did not

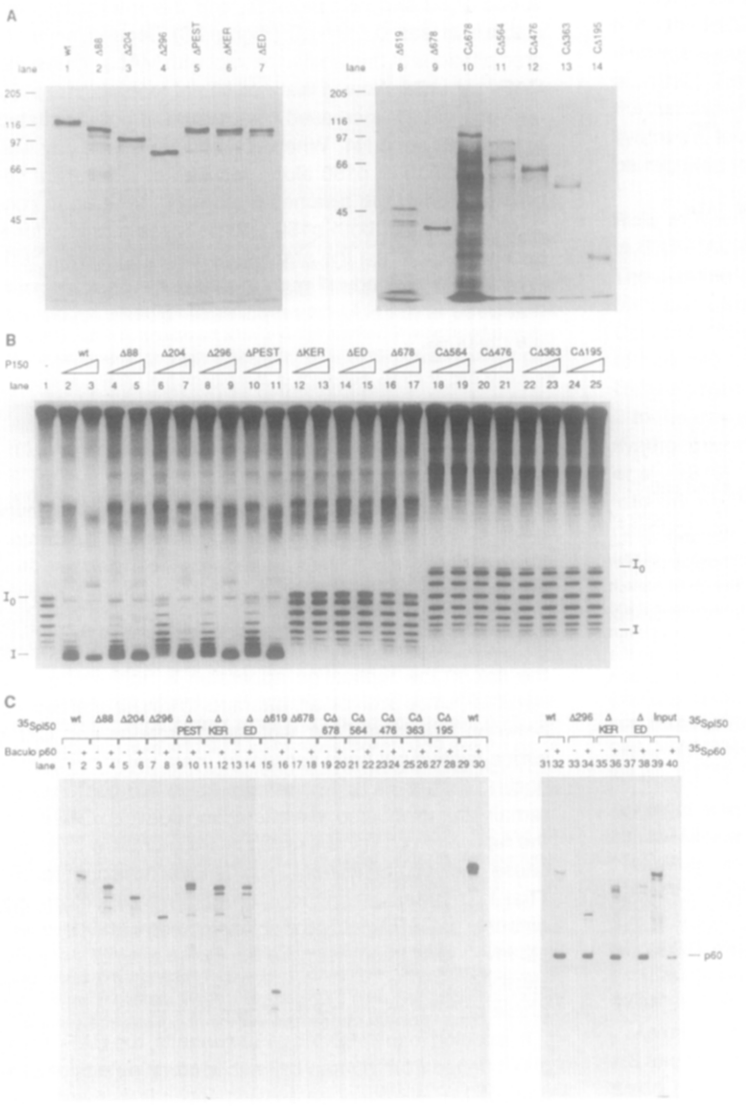

Figure 5. Analysis of p150 Deletion Mutants

(A) In vitro translation of p150 mutants. We analyzed 50 arbitrary units of each full-length product (40 U for Δ619) on a 10% SDS–polyacrylamide gel. Arbitrary units were calculated by Fuji phosphoimager quantitation of full-length protein present in a given volume of each translation. The Δ619 and CΔ678 templates translated particularly poorly, so that these were of insufficient concentration to test in the chromatin assembly assay (see below). We do not know why the CΔ678 protein appears to be particularly susceptible to degradation or premature translational stopping or both.

(B) Chromatin assembly by p150 mutants. SV40 replication reactions (50 μl) were performed with undepleted S100 cytosol extract supplemented with in vitro translated p150 proteins. We used 300 or 600 arbitrary units of in vitro translated wild-type p150 proteins (lanes 2 and 3, respectively). The same amounts of each mutant were used in subsequent pairs of lanes, adjusted for the predicted number of methionines present in the particular proteins so that the same number of molecules was added. Migration of form I negatively supercoiled and form I_0 relaxed monomer circle DNA is indicated.

(C) Coimmunoprecipitation of p150 mutants with p60. Recombinant p60 (0.21 μg; even-numbered lanes, 2–30) was mixed with the indicated in vitro translated p150 proteins and immunoprecipitated with anti-p60 MAb 24 beads. We used 300 arbitrary units of the p150 proteins, except in the following cases in which poor translation required use of less protein: Δ619, 75 U; CΔ678, 63 U. For these precipitation experiments, the amount of in vitro translated proteins was not adjusted for the number of methionines. For lanes 31–38, 600 arbitrary units of in vitro translated p60 was used in the even-numbered lanes in place of purified recombinant p60. Input wild-type in vitro translated p150 (lane 39) and in vitro translated p60 (lane 40) are shown; migration of p60 is indicated.

wt, wild-type.

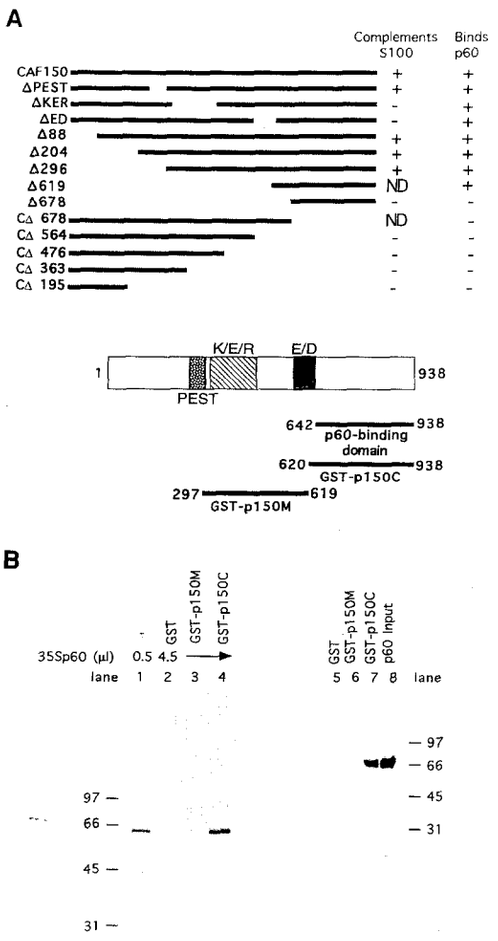

Figure 6. Direct Interaction between p150 C-terminus and p60

(A) (Upper panel) Summary of p150 mapping data. The portions of the p150 protein intact in the various constructs are indicated by the closed line(s) next to the construct names on the left. The plus signs in the column labeled Complements S100 indicate that the given protein performed chromatin assembly in the presence of undepleted S100 cytosolic extracts (data from Figure 5B). The plus signs in the column labeled Binds p60 indicate coprecipitation by anti-p60 antibody in the presence of p60 (data from Figure 5C). ND, not determined (translation of these constructs was too inefficient for testing in the assembly assay). (Lower panel) Domain diagram of p150. The PEST, KER, and ED regions are shown on the same scale as in the upper panel. Also diagrammed are the amino acids in the p60-binding domain and the GST fusion constructs.

(B) GST-fusion protein binding. In vitro translated p60 (4.5 μl, lanes 2–4) or 0.16 μg of recombinant p60 (lanes 5–7) was mixed with 1.2 μg of GST (lanes 2 and 5), GST–p150M (lanes 3 and 6), or GST–p150C (lanes 4 and 7) and precipitated with glutathione–CL4B resin. Proteins eluted from washed beads were separated on a 10% SDS–polyacrylamide gel and analyzed by autoradiography (lanes 1–4) or transferred to nitrocellulose and probed with anti-p60 MAbs 96 and 53 (lanes 5–8). Lane 1 shows 0.5 μl of in vitro translated p60 and lane 8 shows 0.21 μg of recombinant p60 as markers. Molecular mass markers in kilodaltons are indicated on the right.

(Figure 7A, lanes 1 and 2). Also, the anti–acetylated peptide serum precipitated some of the p60 present in the extract, but normal rabbit sera did not (Figure 7A, lanes 3 and 4). Only a subset of the p60 and histones H3 and H4 interact in cytosol extract, because this interaction is dependent on the small amount of endogenous p150 present in the S100 extract (see below). Next, we examined proteins in 293 cell nuclear extracts, where p150 is more abundant. In this case, both the p150 and p60 subunits were in complexes precipitated by the anti–acetylated H4 antisera (Figure 7B, lane 4), but not normal rabbit sera (Figure 7B, lane 5).

In the in vitro DNA replication reactions, addition of CAF-I proteins to the S100 cytosol extract promotes chromatin assembly. We therefore tested whether addition of recombinant p150 and p60 to the S100 cytosol extract would increase the amount of the chromatin assembly factor subunit–histone complexes. First, the anti–acetylated peptide serum, but not control serum, coprecipitated added p150 and p60 proteins, and this interaction was stable in at least 0.5 M NaCl (Figure 7C). Second, immunoprecipitations of the labeled extract with MAbs against the CAF-I subunits showed that addition of recombinant p150 and p60 greatly increased the amount of coprecipitated histones H3 and H4. When the anti-p150 antibody was used, addition of p150 alone resulted in substantial coprecipitation of the histones (Figure 7C, lane 2). In contrast, addition of both p150 and p60 was required for a similar effect when the precipitation was performed with an anti-p60 antibody (Figure 7C, lane 8). This suggests that p150 is the subunit responsible for interaction with the histones.

Discussion

Mechanism of CAF-I Activity

Previous work (Krude and Knippers, 1993) and our own calculations (data not shown) suggest that CAF-I acts stoichiometrically in vitro. We propose that, consistent with a nonenzymatic mechanism, the p150 and p60 subunits act as molecular chaperones involved in directing newly translated histones H3 and H4 to the DNA replication fork. How the link to the replication apparatus is achieved is still not clear, since preliminary attempts to detect interactions between individual DNA replication proteins and CAF-I subunits have not been successful (data not shown), suggesting that important chromatin assembly components remain unknown. Also, the interaction between CAF-I and the replication proteins is probably transient in nature, because nucleosome assembly by CAF-I is noncooperative. That is, assembly reactions containing subsaturating amounts of CAF-I yield product molecules with intermediate levels of negative supercoils, rather than fewer completely assembled molecules (Smith and Stillman, 1989; Krude and Knippers, 1993; Figure 4).

In addition to the p60 and p150 subunits, the CAF-I complex purified from human cell nuclei contains a third subunit, p50. Preliminary data using recently derived poly-

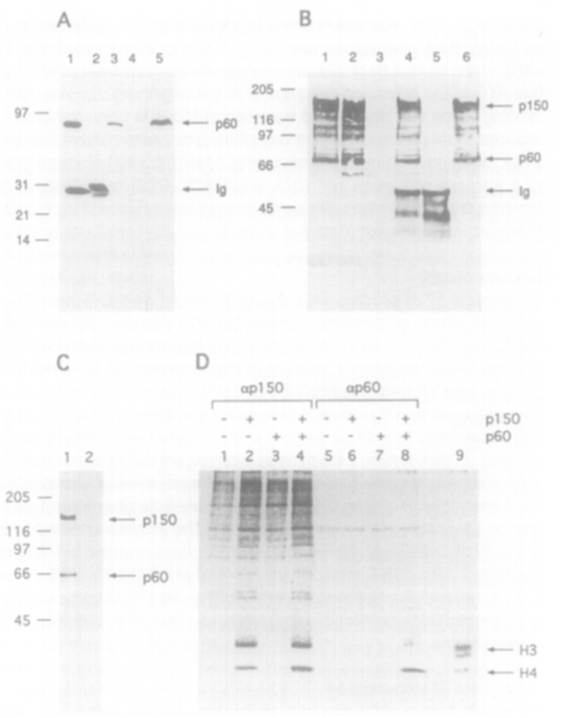

Figure 7. Interactions among Newly Synthesized Histones H3 and H4 and CAF-I Subunits

(A) Endogenous proteins in the 293 cell S100 cytosol extract. Approximately 0.18 mg of ^{14}C-labeled S100 extract protein (~1 × 10^6 cpm) was immunoprecipitated with anti-p60 MAb 24 (lane 1), MAb 12CA5 (lane 2), anti–acetylated H4 rabbit sera (lane 3), or normal rabbit sera (lane 4). Lane 5, unlabeled S100 extract marker. Precipitated proteins were separated on high resolution 18% SDS–polyacrylamide gels (Thomas, 1989), immunoblotted, and probed with anti-p60 MAbs 96 and 53. Ig indicates mouse immunoglobulin light chain that cross-reacts with the secondary antibody.

(B) Endogenous proteins in nuclear extract. Approximately 0.05 mg of protein was either analyzed directly (lane 6) or first immunoprecipitated with 30 μl of anti-p150 MAb 1 (lane 1), 30 μl of anti-p60 MAb 24 (lane 2), MAb 12CA5 (lane 3), anti–acetylated H4 rabbit sera (lane 4), or normal rabbit sera (lane 5). Proteins were separated on a 10% SDS–polyacrylamide gel, immunoblotted, and probed with a mixture of anti-p150 MAbs 1 and 48 and anti-p60 MAbs 96 and 53. Detected proteins migrating between the indicated full-length p150 and p60 species are proteolytic fragments of p150 (data not shown). Ig indicates rabbit immunoglobulin heavy chain that cross-reacts with the secondary antibody.

(C) Coimmunoprecipitation of recombinant p150 and p60 with acetylated H4 in S100 cytosol extract. S100 extract protein (0.72 mg) was supplemented with 0.13 μg of recombinant p150 and 0.1 μg of recombinant p60 and immunoprecipitated with anti–acetylated H4 rabbit sera (lane 1) or normal rabbit sera (lane 2). Proteins were analyzed as in (B).

(D) Coimmunoprecipitation of newly synthesized histones H3 and H4 with recombinant p150 and p60 in S100 cytosol extract. We immunoprecipitated 80 μg of ^{14}C-labeled S100 extract protein (~4.6 × 10^5 cpm) with anti-p150 MAb 1 (αp150, lanes 1–4) or anti-p60 MAb 24 (αp60, lanes 5–8) after addition of 0.2 μg of recombinant p150 (lanes 2, 4, 6, and 8) and 0.15 μg of p60 (lanes 3, 4, 7, and 8). Precipitated proteins were separated on a high resolution 18% SDS–polyacrylamide gel (Thomas, 1989) and visualized on a Fuji phosphoimager. ^{14}C-labeled histone markers are also shown (lane 9).

clonal antisera suggest that this protein is present in the S100 cytosol extract. However, we are currently unable to deplete this protein and thus cannot presently address its function (data not shown). Molecular characterization of the p50 subunit is in progress.

Domains of the p150 and p60 Proteins

Molecular dissection of the p150 protein shows that it contains at least three domains. The C-terminal third of p150 contains a high affinity binding site for the p60 protein, which cannot be deleted without destroying chromatin assembly activity. The middle third of the protein contains highly charged regions that also cannot be deleted without destroying activity, yet such mutants still bind p60, suggesting that the acidic clusters in this region could be involved in interaction with the highly basic histone proteins. Alternatively, this region could be for interaction with other proteins or for correct three-dimensional folding of the protein. The N-terminal third of p150 can be deleted without affecting chromatin assembly activity. This region contains the PEST box, a motif found in many proteins that undergo regulated degradation, such as cyclins (Tyers et al., 1992). The p60 C-terminus also contains such a motif. Further experiments regarding cell-cycle regulation of the synthesis, location, and activity of the CAF-I subunits will be required to address the possible role of these motifs.

Role of Newly Synthesized Histones in CAF-I Activity

The ε-amino groups of lysines near the N-termini of core histones undergo reversible acetylation (reviewed by Csordas, 1990; Turner, 1991). In the nucleus, acetylation of histones assembled into chromatin is correlated with DNaseI hypersensitivity and transcriptional activation.

In the cytoplasm, histone acetylation is found on newly synthesized molecules (Ruiz-Carillo et al., 1975; Jackson et al., 1976). This modification is rapidly lost upon entry into the nucleus and assembly into chromatin unless deacetylases are inhibited by sodium butyrate. The relationship between the cytoplasmic acetylation and chromatin assembly has been most clearly shown in experiments using the unicellular protozoan Tetrahymena. This organism contains two nuclei, a transcriptionally active macronucleus, and a transcriptionally inactive micronucleus. During mating, the micronuclei remain transcriptionally inert while undergoing multiple rounds of DNA synthesis, allowing the study of histone acetylation in the absence of transcription. Pulse-labeled histones from the micronuclei of mating cells showed that newly synthesized histones H3 and H4 were both acetylated (Allis et al., 1985). N-terminal sequence analysis showed that the H4 was specifically acetylated on Lys-4 and Lys-11 (corresponding to residues 5 and 12 of mammalian H4; Chicoine et al., 1986). The same specific pattern of diacetylation has been observed in mammals and Drosophila (Sobel et al., 1994, 1995).

As mentioned above, CAF-I will only assemble histones H3 and H4 derived from cytosolic extracts. Newly synthesized histone H4 in such extracts is in a complex that

contains newly synthesized histone H3, among other proteins (Perry et al., 1993). Our data show that these complexes are bound by the p60 and p150 CAF-I subunits, which subsequently act to deposit the histones onto replicating DNA.

Why would cells use acetylation to target newly synthesized histones for replication-linked chromatin assembly? One possibility is that acetylation plays a role in regulating transcription factor access to nascent chromatin. In this regard, transcription factor TFIIIA can bind reconstituted nucleosomes containing hyperacetylated or trypsinized core histones, but not nucleosomes containing an unmodified histone octamer (Lee et al., 1993), suggesting that acetylation of the histone tails regulates access to nucleosomal DNA. Alternatively, acetylation may be used just as a marker and not because it changes the interaction of histones with DNA. Better characterization of factors that cooperate with CAF-I during DNA replication–linked chromatin assembly will allow for direct examination of this issue.

Experimental Procedures

Purification and Protein Sequencing of CAF-I from Human 293 Cells

Nuclear extracts were prepared from 64 l of 293 cells, and P11 phosphocellulose chromatography was performed as described previously (Smith and Stillman, 1989). The 0.38–0.6 M P11 fraction was dialyzed overnight against chromatography buffer A (25 mM Tris–HCl [pH 7.5], 1 mM EDTA, 10% glycerol, 0.05% NP-40) plus 0.15 M NaCl. All chromatography buffers were freshly supplemented with 0.1 mM DTT and the following protease inhibitors: 1 mM PMSF, 1 mM sodium metabisulfite, 100 μg/ml bacitracin, 1 mM benzamidine, 1 μg/ml each of pepstatin, aprotinin, and leupeptin (all chromatography included these additions). Dialyzed proteins were passed through an ~0.5 ml column of Sepharose–CL4B and then rocked for 1 hr at 4°C with ~2 ml of MAb 24–protein A–Sepharose resin (cross-linked at approximately 5 mg of antibody per milliliter of resin using dimethylpimelimidate, as described by Harlow and Lane, 1988). The resin was then collected into a column, washed with 40 ml of buffer A plus 0.1 M NaCl and then 25 ml of buffer A plus 0.4 M NaCl, and CAF-I was eluted with 3.5 M $MgCl_2$, 25 mM Tris–HCl (pH 7.5), 100 mM NaCl, 0.05% NP-40. The eluate was immediately loaded onto a 0.2 ml hydroxylapatite column (Bio-Rad, HTP grade), which was then washed with solution B (10% glycerol, 0.05% NP-40) plus 10 mM potassium phosphate (pH 7.5). The CAF-I complex was eluted with solution B plus 0.6 M potassium phosphate (pH 7.5). CAF-I was diluted to a conductivity equal to buffer A plus 0.1 M NaCl, loaded onto an ~0.1 ml Q-Sepharose column, and eluted with buffer A plus 0.5 M NaCl.

Human CAF-I (Q-Sepharose peak) was separated on a 10% SDS–polyacrylamide gel, stained with Coomassie blue G (Aldrich), and individual bands were excised. Proteins were digested in situ with lysylendopeptidase; the resulting peptides were separated by reverse phase chromatography and sequenced by Edman degradation as described previously (Bell et al., 1993).

Cloning of p150

A HeLa cell cDNA library constructed in λgt11 was screened with a mixture of MAbs 1 and 48 (anti-p150) and 53 and 96 (anti-p60) raised against CAF-I (Smith and Stillman, 1991b). Two nonoverlapping positives were obtained; one reacted with MAb 1 (termed pTB1), the other with MAb 48 (termed pTB2). No p60-reactive clones were found. The pTB2 cDNA contained poly(A) tail sequences, so the pTB1 insert was used to screen a HeLa cell random-primed λgt10 cDNA library (a gift of R. Tjian) to find missing portions of the cDNA. Four independent positives were obtained (pTB5, 6, 8, and 9), which together overlapped both original inserts. DNA from these inserts was sequenced on both strands with Sequenase II (United States Biochemical). A composite cDNA of 3.15 kb was constructed in pBluescript SK(+) (Stratagene) by fusing the 5′-end sequences of pTB9 to the 3′-end sequences of pTB2 at the unique BssHII restriction site, forming plasmid pPK8. The first ATG in the predicted message is at the beginning of a long open reading frame that would yield a protein of 105 kDa upon translation. Included in this protein are all the peptide sequences obtained during amino acid sequence analysis of purified p150 (Figure 1). Primer extension analysis of human cell RNA and RACE–PCR techniques confirmed that this cDNA includes sequences at or very close to the correct 5′ end of the transcript (data not shown).

Cloning of p60

Degenerate 17 nt primers were designed based on the N- and C-terminal six amino acids of the longest peptide obtained (23 residues; see Figure 2A). RT–PCR reactions using these primers and human 293 cell RNA produced a product of the expected 67 bp size. This product was cloned; several of the isolates contained internal sequences encoding the same amino acids predicted from the peptide sequence data. The 67 bp fragment was labeled and used to probe a human cDNA library. Two positive clones containing hybrid cDNAs were isolated; the two correct halves of the gene were identified by the presence of sequenced peptides in the predicted translation products on either side of an internal EcoRI site. The two halves were fused at the EcoRI site in a pBluescript vector to form plasmid pPK7. The resulting 2.1 kb cDNA encodes a protein of predicted M_r of 61.5 kDa that contains all the sequenced peptides (Figure 2A). Upstream of the predicted initiator methionine, there are no other ATG codons in any reading frame.

Other DNA Manipulations

Constructs for the p150- and p60-producing baculoviruses were made by cleaving pPK7 and pPK8 with HindIII, treating them with Klenow and dNTPs for blunt-end formation, followed by cleavage with EagI. The 3.1 kb fragment containing the p150 cDNA and the 2.1 kb fragment containing the p60 cDNA were separately ligated into pVL1393 (Pharmingen) and digested with SmaI and EagI to form pPK11 and pPK12, respectively. Baculoviruses were generated by cotransfection of Sf9 cells with plasmids and BaculoGold linear viral DNA according to the instructions of the manufacturer (Pharmingen).

The p150ΔPEST, ΔKER, and ΔED internal deletions were made by the Kunkel method, using the *dut⁻ung⁻* Escherichia coli strain BW313 transformed with pPK8 essentially as described previously (Sambrook et al., 1989). Candidate mutants were tested for the presence of a novel restriction endonuclease recognition site generated at the junction (BamHI for ΔPEST and ΔKER; SalI for ΔED), and the correct nucleotide sequence at the deletion breakpoint was confirmed by DNA sequencing.

Oligonucleotides for generating internal deletions were as follows: ΔPEST, 5′-CCAGCCACAC CCCAAGGATC CACGCCCCTC CGCAG-3′; ΔKER, 5′-ACTAAGAAAT TCGTCAAAGG ATCCTGTGGG AAGTTTGCC-3′; ΔED, 5′-GAAGACGGCA CTCATCCGTC GACAGAAACT GAAGGCCAAG G-3′. Oligonucleotides for generating N-terminal deletions were as follows: Δ88, 5′-ATTAATACGA CTCACTATAG GAAACAGACA CCATGGAAAC CAGTATTGGCCAG-3′; Δ204, 5′-ATTAATACGA CTCACTATAG GAAACAGACA CCATGGACAG TTGGAGTGAAGC-3′. These oligonucleotides were used in conjunction with the Stratagene T3 primer in PCR reactions using pPK8 as a template to generate DNA templates for in vitro transcription/translation.

In vitro translation of wild-type p150 protein was performed using construct pPK38. This was made by insertion of the 3.1 kb NcoI–XbaI fragment of pPK8 into the pCITE-1 vector (Novagen). C-terminally truncated p150 proteins were generated by digesting pPK38 with restriction enzymes prior to in vitro transcription/translation as follows: CΔ195, AvaI; CΔ363, AflII; CΔ476, PvuII; CΔ564, BssHII; CΔ678, FspI.

In vitro translation of the p150Δ296 protein was performed using construct pPK49. This was made by insertion of the 2 kb BamHI–EcoRI fragment of the p150ΔPEST plasmid into BamHI–EcoRI-cleaved pCITE-2a (Novagen).

Construct pPK48 for overproduction of GST–p150C was made by insertion of the 1.3 kb NcoI (Klenow-treated)–SacI fragment of pPK8 into NcoI (Klenow-treated), SacI-digested pGEX-KG (Guan and Dixon, 1991).

Construct pPK53 for overproduction of GST–p150M was made by insertion of the 0.9 kb NcoI fragment of pPK49 into the NcoI site of pGEX-KG. Unfused GST protein was overproduced using the pGEX-KG plasmid.

Recombinant Protein Purification

Approximately 2 × 10^7 logarithmically growing Sf9 insect cells were plated on each of ten 150 mm^2 tissue culture flasks. Baculoviruses were added at a multiplicity of infection of ten to each flask, and infection was allowed to proceed for 36 hr at 27°C. Cytoplasmic and nuclear extracts were then prepared essentially as described previously (Smith and Stillman, 1989).

The ammonium sulfate pellet from the p150-containing nuclear extract was resuspended in buffer A plus 0 M NaCl to a final conductivity equal that of buffer A plus 0.15 M NaCl. The extract was then passed through an ~0.5 ml column of Sepharose–CL4B, rocked for 1 hr at 4°C with ~2 ml of MAb 1–protein G–Sepharose (cross-linked as for the MAb 24 resin). The resin was then collected into a column and washed with 40 ml of buffer A plus 0.1 M NaCl and then with 25 ml of buffer A plus 0.3 M NaCl. The bulk of the p150 subunit was eluted with buffer A plus 4 M LiCl. The antibody eluate fraction was immediately loaded onto a 1 ml hydroxylapatite column. The column was washed with 10 ml of buffer A (without EDTA) plus 0.2 M NaCl and then with 10 ml of solution B plus 0.25 M potassium phosphate (pH 7.5). p150 was eluted with solution B plus 0.8 M potassium phosphate. EGTA was added to the p150 fraction to a 0.1 mM final concentration. The p150 protein was diluted with buffer A plus 0 M NaCl to a conductivity equal that of buffer A plus 0.2 M NaCl and loaded onto a small (0.1–0.2 ml) Q-Sepharose column. The column was washed with buffer A plus 0.2 M NaCl; p150 was eluted with buffer A plus 0.5 M NaCl.

Immunoaffinity chromatography of nuclear extracts prepared from Sf9 cells infected with the p60 baculovirus was performed as described for the human CAF-I complex. Then, p60 was loaded onto an ~1 ml hydroxylapatite column. The column was washed with 10 ml of buffer A (without EDTA) plus 0.2 M NaCl and then with 10 ml of solution B plus 10 mM potassium phosphate (pH 7.5). p60 was eluted with solution B plus 0.25 M potassium phosphate (pH 7.5). EGTA was added to the p60 fraction to a 0.1 mM final concentration. The p60 protein was diluted with buffer A plus 0 M NaCl to a conductivity equal that of buffer A plus 0.1 M NaCl and loaded onto a small Q-Sepharose column. The column was washed with buffer A plus 50 mM NaCl; p60 was eluted with buffer A plus 0.3 M NaCl. p60 was dialyzed against buffer A plus 25 mM NaCl plus 20% sucrose, frozen in liquid nitrogen in small aliquots, and stored at −70°C.

DNA Replication Reactions and Product Analysis

SV40 DNA replication reactions using human 293 cell cytosolic extracts, the pSV011 plasmid, and SV40 T antigen overproduced in insect cells were performed and analyzed as described previously (Stillman, 1986; Smith and Stillman, 1989).

For micrococcal nuclease digestion, 300 μl replication reactions were performed for 1 hr at 37°C. These reactions were supplemented with 480 ng histones H2A/H2B purified from 293 cell chromatin (Simon and Felsenfeld, 1979), because the human cytosol extract has substoichiometric amounts of these compared with histones H3 and H4 (data not shown; see also Smith and Stillman, 1989, 1991a). At the end of the 1 hr reaction, $CaCl_2$ was added to a final concentration of 3 mM for reactions performed with undepleted cytosolic extracts; 10 mM $CaCl_2$ was used with p60-depleted extracts. Reactions were then divided into six aliquots of 50 μl each and digested with micrococcal nuclease at 20°C and analyzed by gel electrophoresis as described previously (Smith and Stillman, 1989).

Analysis of In Vitro Translated Proteins

In vitro transcription/translation reactions were performed using the TNT T7 coupled reticulocyte lysate system (Promega) according to the instructions of the manufacturer. Amounts of full-length material for each construct were normalized per volume of lysate by analysis of SDS–polyacrylamide gels on a Fuji phosphoimager. Immunoprecipitations were performed by mixing indicated amounts of in vitro translated proteins or recombinant p60 with 15 μl of a 15% slurry of MAb 24 (Smith and Stillman, 1991b) cross-linked to protein A–Sepharose and 15 μl of a 50% slurry of CL4B (Pharmacia) as carrier resin. The reactions were mixed by inversion at 4°C for 1 hr. The beads were collected by brief pelleting in a microfuge, washed twice with 1 ml of 50 mM Tris–HCl (pH 8.0), 1% NP-40, 0.5 M NaCl, once with 1 ml of 50 mM Tris–HCl (pH 8.0), 1% NP-40, 0.15 M NaCl, and resuspended in SDS–PAGE loading buffer. Proteins were separated on 10% SDS–polyacrylamide gels and autoradiographed.

GST Protein Production and Precipitations

GST and GST fusion proteins were overproduced in E. coli strain BL21(DE3) carrying pLysS. Cultures of 500 ml were grown at 30°C to A_{600} 0.6–0.8. IPTG was added to a final concentration of 0.5 mM, and growth was continued for 4 hr. Cells were harvested and frozen in liquid nitrogen. For lysis, batches of induced cells were resuspended in 25 ml of 20 mM Tris–HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, 0.5% NP-40 at 4°C. All buffers also included 1 mM DTT and PMSF. The lysed cells were sonicated briefly. Debris was pelleted at 10,000 rpm in an SS34 rotor, and the supernatant was mixed with 0.6 ml of glutathione–CL4B resin (Pharmacia) at 4°C for 1 hr. The resin was washed with 10 column volumes of lysis buffer, 10 column volumes of phosphate-buffered saline, and then 2 column volumes of 50 mM Tris–HCl (pH 8.7), 1 mM EDTA, 10% glycerol, 0.05% NP-40, 0.1 M NaCl. GST proteins were then eluted with the latter buffer with 10 mM glutathione freshly added. Pooled protein peaks were dialyzed against chromatography buffer A plus 0.1 M NaCl to remove glutathione, frozen, and stored at −70°C.

For each precipitation, 5 μl of a 75% slurry of glutathione–CL4B resin was mixed with 15 μl of CL4B as carrier resin (Pharmacia). Resins were washed three times with 50 mM Tris–HCl (pH 8.0), 1% NP-40, 150 mM NaCl and resuspended to a volume of 30 μl per reaction in the same buffer. Washed resins were distributed to individual tubes, rocked at 4°C for 1 hr with proteins, and washed twice with 1 ml of 50 mM Tris–HCl (pH 8.0), 1% NP-40, 500 mM NaCl and once with 1 ml of 50 mM Tris–HCl (pH 8.0), 1% NP-40, 150 mM NaCl. Bound proteins were then resuspended in SDS–PAGE sample buffer and analyzed on 10% SDS–polyacrylamide gels.

293 Cell Labeling and Immunoprecipitations

293 cells were pulse-labeled with ^{14}C-lysine and ^{14}C-arginine for 1 hr prior to harvest, and S100 cytosol extracts were prepared as described previously (Smith and Stillman, 1991a) in the presence of 10 mM sodium butyrate. For immunoprecipitations using mouse MAbs, 40–50 μl of a 15% slurry of the antibody covalently coupled to protein A–Sepharose (Pharmacia) was washed three times with 1 ml of wash buffer (25 mM Tris–HCl [pH 8.0], 1 mM EDTA, 1% NP-40, 10 mM sodium butyrate) plus 50 mM NaCl (Figure 7A) or 150 mM NaCl (Figure 7C). Resins were resuspended to 100 μl per precipitation in the same buffer before addition of extract. For immunoprecipitations using rabbit sera, 5–15 μl of a 50% slurry of protein A–Sepharose was mixed with 2.5 μl of rabbit sera at 4°C for 30 min. Unbound proteins were removed by two washes with wash buffer plus 50 mM NaCl (Figure 7A) or 150 mM NaCl (Figure 7B), and resins were then treated as for the mouse antibodies. Indicated amounts of extracts were mixed with the prepared resins for 1 hr at 4°C. Beads were then washed five times with 1 ml of the wash buffer plus 50 mM NaCl (Figure 7A) or four times with wash buffer plus 150 mM NaCl, followed by one time with wash buffer plus 50 mM NaCl (Figure 7B) or four times with wash buffer plus 500 mM NaCl, followed by one time with wash buffer plus 50 mM NaCl (Figure 7C) and were then resuspended in protein gel sample buffer.

Immunoblotting was performed as previously described (Harlow and Lane, 1988). Proteins were detected using ECL reagents (Amersham) according to the instructions of the manufacturer.

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