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Immunological Characterization of Chromatin Assembly Factor I, a Human Cell Factor Required for Chromatin Assembly during DNA Replication *in Vitro**

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Chromatin assembly factor I (CAF-I) is a multisubunit protein complex purified from the nuclei of human cells and required for chromatin assembly during DNA replication *in vitro*. Purified CAF-I promotes chromatin assembly in a reaction that is dependent upon, and coupled with, DNA replication and is therefore likely to reflect events that occur during S phase *in vivo*. In order to investigate the regulation and mechanism of CAF-I and the replication-dependent chromatin assembly process, we have used the purified protein to raise monoclonal antibodies. In this report we describe the characterization of a panel of monoclonal antibodies which recognize different subunits of the CAF-I complex. We use immunoprecipitation analysis to show that CAF-I exists as a multiprotein complex *in vivo* and that some of the polypeptides are phosphorylated. In addition, immunocytochemistry demonstrates that CAF-I is localized to the nucleus of human cells. Finally, monoclonal antibodies directed against the individual subunits of CAF-I immunodeplete chromatin assembly activity from nuclear extracts, confirming that CAF-I is a multisubunit protein required for chromatin assembly *in vitro*.

During each cell cycle not only must all of the DNA in the cell be replicated, but, in addition, the protein structure of chromatin must be accurately reproduced. In S phase, during DNA replication, there is not a protracted period of time where DNA is free of nucleosomes, suggesting that chromatin assembly is tightly coupled to DNA replication *in vivo* (McKnight and Miller (1977); reviewed in DePamphilis and Bradley (1986)). Very little is known, however, about the molecular mechamism of chromatin assembly during DNA replication. To investigate this process, we have used the well characterized cell-free system for SV40 DNA replication (for recent reviews see Stillman (1989) and Chalberg and Kelly (1989)).

SV40 serves as a good model system for the replication and assembly of mammalian chromosomes, since, aside from the virally encoded large T antigen, all of the proteins required for the replication of the viral DNA and its assembly into a chromosome are provided by the host cell (reviewed in DePamphilis and Bradley (1986)). In the cell-free system, a crude cytosol extract from human cells plus purified SV40 T antigen supports the replication of SV40 origin-containing plasmid DNA (Li and Kelly, 1984, 1985; Stillman and Gluzman, 1985; Wobbe *et al.*, 1985). The addition of a nuclear extract promotes assembly of the replicating DNA into a chromatin structure resembling chromatin found *in vivo* (Stillman, 1986). Biochemical fractionation of the nuclear extract has resulted in the identification of a single component that is required for chromatin assembly (Smith and Stillman, 1989). This activity was purified from human cells as a multisubunit protein complex called chromatin assembly factor I, CAF-1[1] (Smith and Stillman, 1989). Purified CAF-I will substitute for the crude nuclear extract in the chromatin assembly reaction described above. Chromatin assembly by CAF-I is dependent upon DNA replication and is therefore likely to reflect the process of chromatin assembly that occurs during DNA replication *in vivo*.

To begin to elucidate the mechanism and regulation of CAF-I, the purified protein was used to raise monoclonal antibodies. In this report, the characterization of a panel of these monoclonal antibodies which recognize individual subunits of the CAF-I complex is described. These results confirm that CAF-I is indeed a multiprotein complex but further demonstrate that it is a nuclear phosphoprotein that is essential for chromatin assembly *in vitro*.

MATERIALS AND METHODS

Monoclonal Antibodies—A highly purified fraction of CAF-I, prepared as described previously (Mono Q, fraction 6 of the purification, Smith and Stillman (1989)), was used to immunize a BALB/c mouse. The mouse was immunized three times during a four week period with purified CAF-I antigen (5 μg of each CAF-I polypeptide per injection) in complete Freund's adjuvant (primary injection) or incomplete Freund's adjuvant (subsequent injections), intraperitoneally. This was followed by two injections (one intraperitoneal and the other intravenous) of 5 μg each of purified CAF-I antigen in phosphate-buffered saline (PBS). Three days later the spleen cells were fused (Kohler and Milstein, 1975) with mouse myeloma cells, NSI/Ag4-1 (Kohler *et al.*, 1976). Hybridoma clones were selected in hypoxanthine/azaserine medium. Hybridoma supernatants were initially screened for their ability to bind to purified CAF-I antigen which had been immobilized onto nitrocellulose using a dot blot procedure (Hawkes, 1986). The second and third screens were for those antibodies that recognized CAF-I polypeptides in an immunoblotting analysis (described below) using purified CAF-I antigen and/or those that could immunoprecipitate CAF-I polypeptides from [^{35}S] methionine-labeled cells under the denaturing conditions described below. The clones of interest were subcloned by the method of limiting dilution and single cell cloning. The monoclonal antibodies were isotyped using the commercially available kit (Baxter Health Care Corp., Pandex Division, Mundelein, IL). All the monoclonal antibodies characterized were mouse IgG1$_a$.

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[1] The abbreviations used are: CAF-I, chromatin assembly factor I; mAb, monoclonal antibody; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate.

The monoclonal antibodies used as controls in the experiments were: pAb 419, a monoclonal antibody directed against SV40 T antigen, previously called L19 (Harlow *et al.*, 1981); mAb p70-9, a monoclonal antibody directed against a 70-kDa polypeptide in replication factor A (Fairman and Stillman, 1988; Din *et al.*, 1990); and mAb 1644, a monoclonal antibody against human DNA polymerase α, originally designated as SJK 287-38 (Tanaka *et al.*, 1982).

A polyclonal mouse antiserum against CAF-I was obtained by bleeding the hyperimmune mouse (described above), after removing the spleen.

Immunoblotting—protein samples were subjected to SDS-polyacrylamide gel electrophoresis according to the procedure of Laemmli (1970), and transferred to nitrocellulose using a dry electroblotting apparatus (American Bionetics Inc., Emeryville, CA). All incubations were carried out at room temperature. Nitrocellulose filters were blocked for 60 min in 3% bovine serum albumin (w/v) in pre-coat buffer (10 mM $NaPO_4$, pH 7.4, 150 mM NaCl, and 0.5% Nonidet P-40). Filters were incubated overnight with hybridoma supernatants and then washed in pre-coat buffer for 15 min, followed by a 15-min wash in buffer containing 2 M urea, 1% Nonidet P-40, and 100 mM glycine, and two more 15-min washes in pre-coat buffer. Nitrocellulose filters were then incubated with a 1:200 dilution of rabbit anti-mouse horseradish peroxidase-conjugated immunoglobulins (Dako Corporation, Carpinteria, CA), for 2 h. Filters were then washed three times for 15 min each in pre-coat buffer, followed by three 5-min washes in PBS. The second antibody was detected by incubation of the filters with a developing solution prepared as follows. A saturated solution of *o*-dianisidine (Sigma) in ethanol was prepared. This was diluted 1:100 into PBS, and H_2O_2 (30%) was added (1:10,000).

Cell Culture—HeLa cells and 293 cells were grown at 37 °C as subconfluent monolayer cultures on 100-mm tissue culture plates in Dulbecco's modified Eagles medium supplemented with 5% fetal calf serum or 10% calf serum, respectively.

Cell Staining—Immunocytochemistry was performed on HeLa cells. All incubations were done at room temperature. Cells were formaldehyde-fixed by incubation in 3.7% formaldehyde for 15 min followed by permeabilization with 0.2% Triton X-100 for 2 min. Cells were methanol/acetone-fixed by incubation in a solution containing 50% methanol, 50% acetone for 5 min. Fixed cells were incubated overnight with hybridoma supernatants and rinsed several times with PBS, followed by a 2-h incubation with a 1:200 dilution of rabbit anti-mouse horseradish peroxidase-conjugated immunoglobulins (Dako Corporation). The second antibody was detected as described above for the immunoblotting procedure.

Labeling of 293 Cells—293 cells were preincubated in methionine-free Dulbecco's modified Eagle's medium supplemented with 2% dialyzed calf serum for 30 min at 37 °C. Cells were then labeled for 4 h at 37 °C with 500 μCi of [^{35}S]methionine (Translabel, ICN Radiochemicals, Irvine, CA) in 1 ml of the same medium per 100-mm dish. For ^{32}P-labeling, 293 cells were preincubated in phosphate-free Dulbecco's modified Eagle's medium supplemented with 2% dialyzed calf serum for 30 min at 37 °C. Cells were then labeled for 4 h at 37 °C with 1 mCi of [^{32}P]orthophosphate in 1 ml of the same medium per 100-mm dish. The labeled cells were lysed, and immunoprecipitations were performed as described below.

Immunoprecipitation—[^{35}S]Methionine- or [^{32}P]phosphate-labeled human 293 cells were washed three times with PBS, scraped with a rubber policeman, and collected by centrifugation. For denaturing conditions, cells were lysed with 500 μl of RIPA buffer (50 mM Tris, pH 8.0, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 150 mM NaCl, and 1 mM phenylmethylsulfonyl fluoride), for each 100-mm dish. The sample was incubated on ice for 10 min with 50 μl of a solution containing 0.5 M Tris, pH 7.2, 50 mM $MgCl_2$, 0.5 mg/ml RNase, and 1.0 mg/ml DNase. Cells were homogenized through a 25-gauge syringe needle and cleared by centrifugation. Immunoprecipitations were carried out by incubating the cell lysate with 200 μl of monoclonal antibody supernatant for 60 min on ice, followed by incubation with 1 μl of rabbit anti-mouse immunoglobulins (Dako Corporation) on ice for 30 min. The samples were then cleared of debris by centrifugation. The immunocomplex was precipitated after incubation with 50 μl of 1:1 slurry of protein A-Sepharose (Pharmacia LKB Biotechnology Inc.) in RIPA buffer. Complexes were washed six times with RIPA buffer, and then proteins were eluted from the protein A-Sepharose beads by boiling in 50 μl of Laemmli sample buffer for 3 min and analyzed by SDS-polyacrylamide gel electrophoresis. Gels were fixed in a solution containing 40% methanol and 10% acetic acid for 15 min, washed in H_2O two times for 10 min each, treated with 1 M sodium salicylate for 30 min, and dried for autoradiography. For non-denaturing conditions, immunoprecipitations were performed exactly as described above except Nonidet P-40 buffer (50 mM Tris, pH 8.0, 1% Nonidet P-40, and 1 mM phenylmethylsulfonyl fluoride) containing 150 or 500 mM NaCl was used in place of RIPA buffer.

Purification of Monoclonal Antibodies—Antibodies were purified from ascites fluid which was raised by injecting nude mice with hybridoma supernatants. 4 ml of ascites fluid (approximately 5 mg/ml of immunoglobulins), was applied to a 1-ml protein A-Sepharose (Pharmacia) column equilibrated in buffer containing 3 M NaCl and 50 mM sodium borate (pH 8.9). The sample was reapplied four times and the column washed with 20 volumes of equilibration buffer. The immunoglobulins were eluted with 0.1 M glycine (pH 3.0). Protein containing fractions were pooled and dialyzed overnight against PBS. The protein concentration was determined by the method of Bradford (1976) using bovine immunoglobulins as a standard.

Immunodepletion—Purified antibodies were conjugated at 3–4 mg/ml of packed Sepharose beads. Antibodies were incubated with cyanogen bromide-activated Sepharose 2B (Sigma) at 4 °C for 3 h. The beads were then removed by centrifugation and washed several times with PBS. Unbound sites were blocked by incubating overnight with 1 M ethanolamine (pH 8.3). Beads were removed by centrifugation, washed extensively with PBS, and resuspended as a 1:1 slurry in PBS.

For immunodepletion, 20 μl of packed immunobeads were mixed with 50 μl of the nuclear extract, prepared as described previously (nuclear extract/ammonium sulfate pellet, fraction 1 of the purification, Smith and Stillman, 1989). Samples were incubated on ice for 1 h with occasional mixing, and the beads were removed by centrifugation. 10 μl of the supernatant was added to a standard SV40 replication reaction carried out exactly as described previously (Smith and Stillman, 1989). The DNA products were isolated and analyzed by electrophoresis through a 1% agarose gel as described (Smith and Stillman, 1989).

Protein samples were processed for immunoblotting. After immunodepletion, bound proteins were eluted off the immunobeads by incubating in 30 μl of Laemmli sample buffer at 68 °C for 5 min. The depleted supernatants were incubated in Laemmli buffer at 68 °C for 5 min. An amount corresponding to approximately 33% of the total depleted supernatant (15 μl) or 33% of the total protein bound to the beads (10 μl) was subjected to SDS-polyacrylamide gel electrophoresis as described by Laemmli (1970). The gels were then immunoblotted as described above or stained with silver according to the method of Wray *et al.* (1981).

RESULTS

Classification of CAF-I Monoclonal Antibodies—CAF-I was purified from human 293 cell nuclei as described previously (Smith and Stillman, 1989) and used to immunize a mouse for the production of monoclonal antibodies. The polypeptide composition of purified CAF-I is shown in Fig. 1*A*, *lane 1*. CAF-I consists of a large subunit with apparent molecular mass of 150,000 (150 kDa), three polypeptides centered at 60 kDa, and a small subunit of 50 kDa (henceforth called p150, p60, and p50, respectively). Eight independent monoclonal antibodies that recognize individual CAF-I subunits have been identified. Immunoblot analysis using these monoclonal antibodies as probes is presented in Fig. 1*B*. Two classes of monoclonal antibodies were obtained; the first class reacted with p150 and the second class with the set of polypeptides designated as p60. Immunoblot analysis further showed that all of the monoclonal antibodies directed against p60 reacted with multiple polypeptides, suggesting that the 60-kDa triplet, identified in the original purified CAF-I, may constitute a related set of proteins (see below). Most of the monoclonal antibodies directed against p60 cross-reacted very weakly with a polypeptide of about 50 kDa (data not shown). It is not known at this time if this band corresponds to the p50 contained in the purified CAF-I complex or if it is simply a minor proteolytic breakdown product of p60 and unrelated to the p50 subunit of CAF-I. Monoclonal antibodies which reacted exclusively with p50 were not obtained in this monoclonal antibody screen. The eight monoclonal antibodies de-

scribed here appear to be independent isolates based upon the size of their light chains (data not shown) or their different specificites in immunoblotting, immunoprecipitation, and intracellular staining analyses. Characterization of a subset of the monoclonal antibodies will be described in detail below and a summary of the properties of all eight monoclonal antibodies is presented in Table I.

Cellular Localization of CAF-I—The monoclonal antibodies were used to determine the intracellular localization of CAF-I. When monoclonal antibody 1, which recognizes p150 (mAb p150-1) was used to stain formaldehyde-fixed human Hela cells, a nuclear strong staining pattern was observed (Fig. 2*A*). Although the staining pattern was observed in all cells of the population, the intensity of the stain varied from cell to cell within the population. When the same antibody was used to stain methanol/acetone-fixed cells, it stained nuclei very weakly and the staining was observed in a fraction of the cells (Fig. 2*A*). Monoclonal antibody 24, which recognizes p60 (mAb p60-24), produces a staining pattern similar to that shown for mAb p150-1 (Fig. 2*B*). In contrast, however, a strikingly different pattern was obtained with a subset of the monoclonal antibodies directed against p60. A representative example is shown in Fig. 2*C* using mAb p60-3. Like the other monoclonal antibodies, mAb p60-3 displayed a strong nuclear stain on formaldehyde-fixed cells (Fig. 2*C*), but in addition to the nuclear stain, a polar perinuclear stain was observed. Interestingly, while the nuclear staining pattern changed in methanol/acetone-fixed cells, as it did with the other monoclonal antibodies, the perinuclear stain was not affected (Fig. 2*C*). As a control, a monoclonal antibody directed against SV40 T antigen, which is not found in Hela cells, did not stain methanol/acetone- or formaldehyde-fixed cells (Fig. 2*D*).

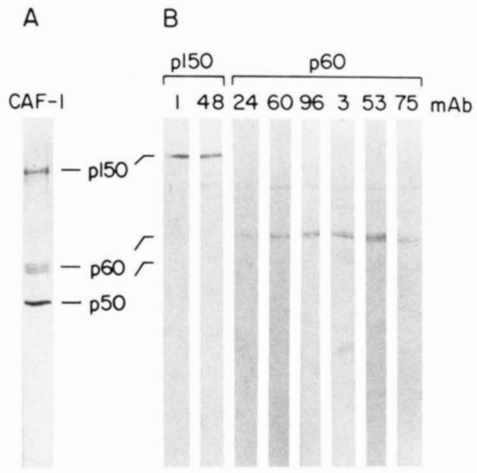

FIG. 1. **Immunoblot analysis of CAF-I monoclonal antibodies.** *A*, purified CAF-I (glycerol gradient purified; Smith and Stillman (1989)) was subjected to electrophoresis through a 12.5% SDS-polyacrylamide gel, and the proteins were visualized by silver staining. *B*, a partially purified fraction of CAF-I (Mono Q, fraction 6 of the purification, Smith and Stillman (1989)) was fractionated on a 12.5% SDS-polyacrylamide gel and the proteins transferred to nitrocellulose. The nitrocellulose was then cut into strips and probed individually with each CAF-I mAb, followed by incubation with rabbit anti-mouse horseradish peroxidase-conjugated immunoglobulins. The immunoblots were developed as described under "Materials and Methods." The positions of the CAF-I subunits, p150, p60, and p50, are shown.

CAF-I Polypeptides Are Complexed in Vivo—The monoclonal antibodies were tested for their ability to immunoprecipitate each of the CAF-I polypeptides. In these initial experiments immunoprecipitations were performed using [^{35}S] methionine-labeled 293 cell extracts prepared in RIPA buffer. As a positive control, a polyclonal mouse antiserum against CAF-I was used to immunoprecipitate the CAF-I polypeptides. As shown in Fig. 3*A*, *lane 1*, this antiserum immunoprecipitated the p60 and p150 subunits of CAF-I. Normal mouse serum did not immunoprecipitate these proteins (data not shown). Fig. 3*A*, *lane 2* shows that the monoclonal antibody, mAb p60-24 immunoprecipitated a polypeptide of approximately 60 kDa. The p150 subunit of CAF-I was immunoprecipitated from [^{35}S]methionine-labeled 293 cells using mAb p150-1 (Fig. 3*A*, *lane 3*). The control antibody, mAb p70-9, a monoclonal antibody directed against a 70-kDa polypeptide of replication factor A did not immunoprecipitate the CAF-I polypeptides but did precipitate the expected 70-kDa replication factor A protein (Fig. 3*A*, *lane 4*).

Under the conditions used for the immunoprecipitation analysis shown in Fig. 3*A*, p60 did not appear to be complexed with p150, since both polypeptides were not coimmunoprecipitated with the monoclonal antibodies (*i.e.* mAb p60-24 did not coimmunoprecipitate p150). We previously determined that CAF-I was a multisubunit protein complex, because the set of proteins (shown in Fig. 1*A*, *lane 1*) fractionated together with chromatin assembly activity over a wide range of chromatographic steps. This suggested that the extract preparation conditions shown in Fig. 3*A* might disrupt this complex. We therefore performed the immunoprecipitation analysis under less stringent conditions to try and preserve the complex. [^{35}S]Methionine-labeled 293 cell extracts were prepared in Nonidet P-40 buffer containing 150 mM NaCl (Fig. 3*B*, *lanes 1–4*). Under these conditions, the polyclonal antibody precipitated all the CAF-1 subunits, including the p50 subunit (*lane 1*), although with the control antibody there was considerable background (*lane 4*). Under these low salt conditions, the specificity of the mAb p60-24 and mAb p150-1 precipitations was difficult to see, particularly for the p60 and p50 proteins (Fig. 3*B*, *lanes 2* and *3*).

TABLE I

Immunological properties of CAF-I monoclonal antibodies

A summary of the properties of CAF-1 monoclonal antibodies. A + indicates reaction in the indicated assay. The blank spaces under immunodepletion indicate not tested.

mAb	CAF-I subunit	Immunoblot	Intracellular localization	Immunoprecipitation	Immunodepletion
1	p150	+	Nuclear	+	+
48	p150	+	Nuclear	−	
24	p60	+	Nuclear	+	+
60	p60	+	Nuclear	−	
96	p60	+	Nuclear	−	
3	p60	+	Nuclear/perinuclear	+	
53	p60	+	Nuclear/perinuclear	+	
75	p60	+	Nuclear/perinuclear	+	

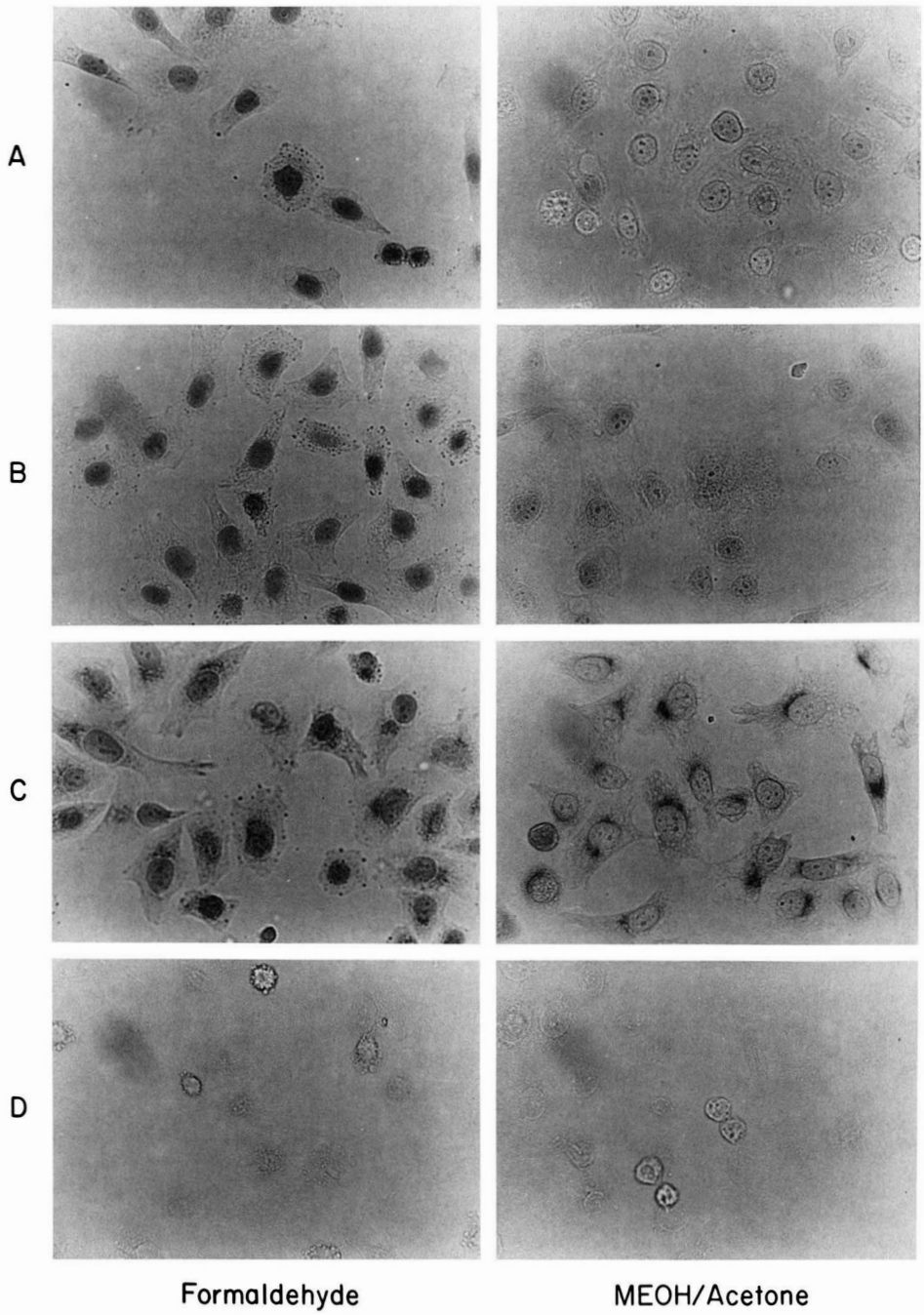

FIG. 2. **Intracellular localization of CAF-I.** Hela cells were fixed with formaldehyde or methanol/acetone (*MEOH/Acetone*) and stained with the following monoclonal antibodies; mAb p150-1 (*A*), mAb p60-24 (*B*), mAb p60-3 (*C*), or pAb 419 (*D*), followed by incubation with rabbit anti-mouse horseradish peroxidase-conjugated immunoglobulins. The stain was developed as described under "Materials and Methods."

The less stringent buffer conditions used in this experiment appeared to stabilize the complex but, at the same time, increased the contaminating background proteins. To reduce this background, [^{35}S]methionine-labeled 293 cell extracts were prepared in Nonidet P-40 buffer containing 500 mM NaCl (Fig. 3*B*, *lanes 5–8*). Under these conditions the polyclonal antisera clearly co-precipitated all subunits, the mAb p60-24 specifically immunoprecipitated the p50, p60 and p150 proteins (*lane 6*), whereas the control, mAb p70-9, did not immunoprecipitate these polypeptides (*lane 8*). Under these high salt conditions, mAb p150-1 was negative in an immunoprecipitation analysis (*lane 7*) and only precipitated the weaker background bands present in the control (*lane 8*). Moreover, as shown in Fig. 3*B*, *lane 6*, under these nondenaturing conditions, mAb p60-24 specifically immunoprecipitated a polypeptide of 50 kDa (note the lower band of the doublet migrating at the 50-kDa position), whereas the control, mAb p70-9, did not (*lane 8*). However, additional experiments will be required to determine if this 50-kDa polypeptide corresponds to the p50 subunit of CAF-I. Nonetheless, these data suggest that the p150 and p60 subunits of CAF-I are complexed *in vivo*.

Immunodepletion of Chromatin Assembly Activity in Vitro—To confirm that the polypeptides designated as CAF-I were, in fact, required for chromatin assembly activity *in vitro*, we sought to use the CAF-I monoclonal antibodies in an immunodepletion analysis. As described previously, CAF-I activity was initially identified in a crude nuclear extract that, when added to a cytosol replication extract, promoted chromatin assembly on replicating DNA (Stillman, 1986; Smith and Stillman, 1989). Fig. 4 shows a DNA product analysis of a series of replication reactions containing cytosol replication extract, SV40 T antigen, SV40 origin-containing plasmid, and nucleoside triphosphates, including [α-^{32}P]dATP. The auto-

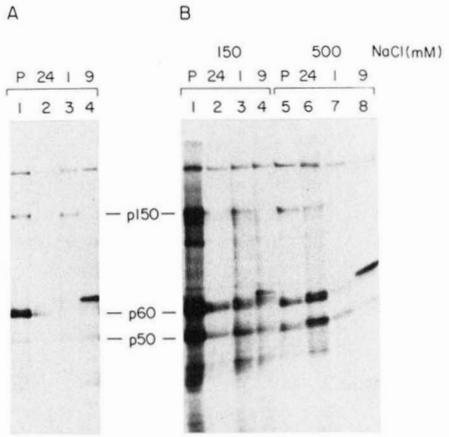

FIG. 3. **Immunoprecipitation of [^{35}S]methionine-labeled CAF-I polypeptides.** [^{35}S]methionine-labeled 293 cell lysates were prepared under (*A*) denaturing conditions (in RIPA buffer) or (*B*) non-denaturing conditions (in Nonidet P-40 buffer containing 150 or 500 mM NaCl) and immunoprecipitated with polyclonal mouse antiserum against CAF-I (*P*) or the following monoclonal antibodies; mAb p60-24 (*24*), mAb p150-1 (*1*), or the control, mAb p70-9 (*9*). Immunoprecipitated proteins were separated on a 10% SDS-polyacrylamide gel and visualized by fluorography. The positions of the CAF-I subunits, p150, p60, and p50, are indicated.

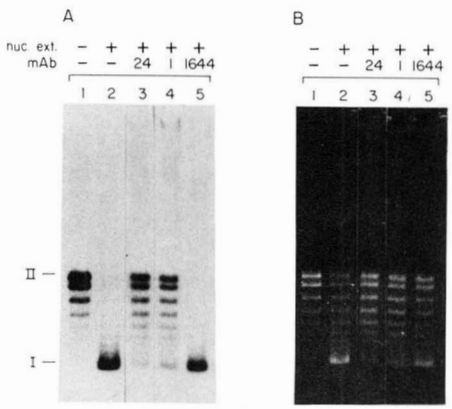

FIG. 4. **Immunodepletion of chromatin assembly activity.** SV40 DNA replication reactions were carried out in the absence (*lane 1*), or presence (*lanes 2–5*) of nuclear extract. Nuclear extract was not precleared (*lane 2*) or was precleared with the following immunobeads; mAb p60-24 (*lane 3*), mAb p150-1 (*lane 4*), or mAb 1644 (*lane 5*). The DNA products were isolated and subjected to electrophoresis in a 1% agarose gel. *A* shows the autoradiograph and *B* the ethidium bromide-stained gel. The positions of *Form I* and *Form II* DNA are shown to the *left*.

radiograph in Fig. 4A shows that DNA replicated in the absence of nuclear extract migrated as a covalently closed relaxed monomer circle DNA (*lane 1*), whereas the addition of the nuclear extract promoted the negative supercoiling of the replicated DNA (*lane 2*). This negative supercoiling reflects assembly of the replicated DNA into chromatin (Stillman, 1986; Smith and Stillman, 1989). The gel was also stained with ethidium bromide prior to autoradiography to visualize the unreplicated DNA (Fig. 4*B*). A comparison of the replicated DNA (Fig. 4*A*, *lane 2*) with the total input DNA (Fig. 4*B*, *lane 2*), demonstrated that all of the replicated DNA was supercoiled, whereas the bulk of the input DNA remained relaxed. This confirms that chromatin assembly in this system occurs preferentially if not exclusively on replicated DNA.

Preliminary experiments demonstrated that neither mAb p150-1 or mAb p60-24 inhibited replication dependent chro-

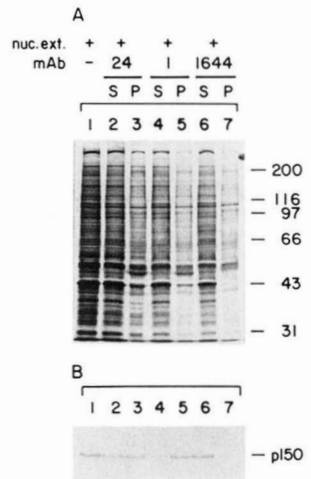

FIG. 5. **Analysis of the proteins in the immunodepleted extracts.** Nuclear extract was preincubated with the specific immunobeads containing the indicated monoclonal antibody covalently attached to Sepharose and then fractionated into supernatants (*S*) and pellets (*P*). A sample of the protein was subjected to electrophoresis through 10% SDS-polyacrylamide gels and visualized by silver staining (*A*) or transferred to nitrocellulose and immunoblotted with mAb p150-1 (*B*) as described under "Materials and Methods." Nuclear extract was not preincubated (*lane 1*) or was preincubated with the following immunobeads; mAb p60-24 (*lanes 2* and *3*), mAb p150-1 (*lanes 4* and *5*), mAb 1644 (*lanes 6* and *7*) and separated into supernatant (*S*) and pellet (*P*) fractions. Note that the precipitates were not washed prior to electrophoresis, and consequently, the silver stain of the gel shows many nuclear proteins.

matin assembly *in vitro* if they were added directly to the reaction (data not shown). Therefore, each of the monoclonal antibodies were covalently coupled to Sepharose beads and immunodepletion was performed by preincubating the nuclear extract with the immunobeads on ice for 60 min. Following the preincubation, the immunobeads were removed by centrifugation and the depleted supernatant was then added to the replication reaction. As shown in Fig. 4, preclearing of the nuclear extract with mAb p60–24 beads (*lane 3*) or mAb p150–1 beads (*lane 4*) depleted the extract of most of the chromatin assembly activity. In contrast, preclearing of the nuclear extract with Sepharose beads coupled to a monoclonal antibody directed against human DNA polymerase α (mAb 1644) had no effect (*lane 5*). Note that in this case, polymerase α for replication was provided by the cytosol replication extract.

To confirm that the immunodepletion actually removed the CAF-I polypeptides from the nuclear extract, the depleted extracts were analyzed by immunoblotting. After the preincubation step, proteins contained in the depleted supernatants or proteins bound to the immunobeads were analyzed by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with mAb p150-1. Direct analysis of the proteins in each sample by silver staining revealed few, if any, differences (Fig. 5*A*). In contrast, however, immunoblot analysis (Fig. 5*B*) showed that the mAb p60-24-depleted (*lane 2*) or mAb p150-depleted (*lane 4*) depleted supernatants, which were both inactive for chromatin assembly, contained reduced amounts of the p150 subunit of CAF-I. In contrast, the mAb 1644-depleted supernatant, which was still active in chromatin assembly, showed no reduction in the amount of p150 (*lane 6*). Accordingly, p150 was found bound to the mAb p60-24 immunobeads (*lane 3*) and the mAb p150-1 immunobeads (*lane 5*) but not the mAb 1644 immunobeads (*lane 7*). These data confirm that p60 and p150 are part of the CAF-I complex

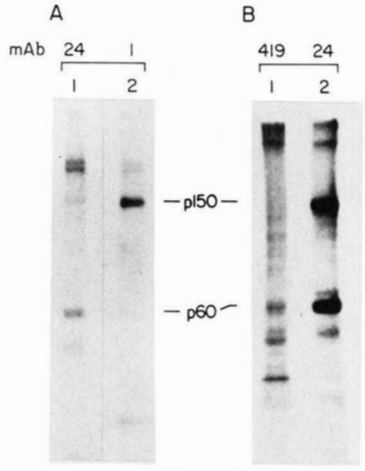

FIG. 6. **Immunoprecipitation of [^{32}P]phosphate-labeled CAF-I polypeptides.** *A*, [^{32}P]phosphate-labeled 293 cell lysates were prepared under denaturing conditions (in RIPA buffer) and immunoprecipitated with mAb p60-24 (*lane 1*) or mAb p150-1 (*lane 2*). *B*, [^{32}P]phosphate-labeled 293 cell lysates were prepared under non-denaturing conditions (in Nonidet P-40 buffer containing 500 nM NaCl) and immunoprecipitated with pAB 419 (*lane 1*) or mAb p60-24 (*lane 2*). Immunoprecipitated proteins were separated by electrophoresis through 10% SDS-polyacrylamide and autoradiographed. The positions of the CAF-I subunits p150 and p60 are shown.

and demonstrate that they are required for chromatin assembly activity *in vitro*.

Post-translational Modification of CAF-I—As shown in the immunoblot analysis presented in Fig. 1*B*, all of the monoclonal antibodies directed against the p60 subunit of CAF-I reacted with multiple polypeptides, suggesting that these proteins shared a common epitope. In contrast, however, immunoprecipitation analysis of [^{35}S]methionine-labeled cells with the same monoclonal antibodies yielded only one or possibly two polypeptides (Fig. 3*A*, *lane 1* and data not shown). These data suggested the possibility that the multiple polypeptides were a result of a post-translational modification, such as phosphorylation, which was not being detected in the [^{35}S] methionine-labeled cell extracts. To test this possibility, [^{32}P] phosphate-labeled 293 cell extracts were prepared under denaturing conditions (in RIPA buffer). As shown in Fig. 6*A*, *lane 1*, a ^{32}P-labeled protein of approximately 60 kDa was immunoprecipitated with mAb p60-24. Interestingly, immunoprecipitation with mAb p150-1 (Fig. 6*A*, *lane 2*), revealed that the p150 subunit of CAF-I was also phosphorylated. When [^{32}P]phosphate-labeled 293 cell extracts were prepared under non-denaturing conditions (in Nonidet P-40 buffer containing 500 mM NaCl), mAb p60-24 immunoprecipitated ^{32}P-labeled p60 and p150 (Fig. 6*B*, *lane 2*), whereas the control, pAb 419, a monoclonal antibody directed against SV40 T antigen, did not immunoprecipitate these polypeptides (Fig. 6*B*, *lane 1*). These data show that both the p60 and the p150 subunits of CAF-I are phosphorylated in the cell and again confirm that these subunits are complexed *in vivo*.

DISCUSSION

In this report we describe the isolation of a panel of monoclonal antibodies directed against the human replication-dependent chromatin assembly factor, CAF-I. As a first step toward investigating the mechanism and regulation of CAF-I, we have used these monoclonal antibodies to characterize some of the properties of CAF-I *in vivo*. The classification of the eight monoclonal antibodies as independent isolates is based upon their immunochemical properties, which are summarized in Table I. In cases where the monoclonal antibodies behaved identically in all assays tested, they were designated as independent isolates based upon differences in the migration of the light and heavy chains of the purified immunoglobulins during SDS-polyacrylamide gel electrophoresis and/or subtle differences in the pattern obtained in the immunoblot analysis.

Cell staining analysis with all of the monoclonal antibodies showed an intranuclear localization of CAF-I. This was not surprising because the protein was initially purified from nuclei, and in addition, a nuclear localization pattern is consistent with CAF-I function *in vitro*; replication coupled chromatin assembly. A strong nuclear staining pattern was obtained only after cells were fixed with formaldehyde. Interestingly, under these conditions, although CAF-I was present in the nucleus of all the cells in the population, the intensity of the stain varied from cell to cell within the population. Immunostaining of cells fixed with methanol/acetone showed only a very weak nuclear stain and only in a fraction of the total cell population. The cells used in this study represent an actively growing unsynchronized population and thus, should contain cells in all stages of the cell cycle. This raises the intriguing possibility that the heterogeneous staining pattern reflects differences in the amount or localization of CAF-I during the cell cycle, although extensive cell cycle studies will be required to investigate this further.

Surprisingly, in addition to the nuclear staining pattern described above, a perinuclear stain was observed using a subset of the CAF-I monoclonal antibodies directed against p60. The polar, perinuclear staining pattern is reminiscent of proteins that localize to the Golgi apparatus and/or endoplasmic reticulum; however, electron microscopic analysis will be required to determine this. That these monoclonal antibodies actually recognized the p60 subunit of the CAF-I complex was confirmed by using immunoprecipitation analysis to demonstrate that each mAb that showed a perinuclear staining pattern also coimmunoprecipitated the p150 subunit of the CAF-I complex (data not shown). The possibility still exists, however, that these monoclonal antibodies simply recognize a common epitope shared by the CAF-I p60 subunit and an unrelated perinuclear protein. This can be addressed by mapping the epitopes that these monoclonal antibodies are directed against. If this perinuclear antigen is in fact CAF-I, it will be interesting to determine if this localization reflects a storage, modification, or assembly site for CAF-I.

The monoclonal antibody screen described in this paper yielded isolates that recognized two of the three components of the CAF-I complex: the p60 triplet and the p150 subunit. We did not, however, obtain monoclonal antibodies against the p50 subunit. Although the protein preparation used to immunize the mouse contained similar amounts of all three components, an immunoprecipitation analysis using the polyclonal mouse antiserum showed only a very weak response to p50 (see Fig. 3*A*, *lane 1*), which could explain why no monoclonal antibodies were obtained. The demonstration, however, that a 50-kDa peptide was coimmunoprecipitated by mAb p60-24 does suggest that p50 is contained in the CAF-I complex. Further characterization of the p50 subunit will await the isolation of monoclonal antibodies which specifically react with it and/or two-dimensional gel analysis of the proteins in the CAF-I complex.

The initial designation of CAF-I as a multisubunit protein complex was based upon cofractionation of a set of polypeptides with chromatin assembly activity (Smith and Stillman, 1989). The use of the monoclonal antibodies directed against CAF-I in the immunodepletion analysis described here served to confirm that p60 and p150 are contained in a complex

which is required for chromatin assembly activity *in vitro*. Interestingly, while mAb p150-1 completely depleted the nuclear extract of p150, mAb p60-24 effected only a partial depletion of p150. Yet, in both cases, the chromatin assembly activity was depleted. Immunodepletion with mAb p60-24 may have disrupted the CAF-I complex, leaving inactive p150 in the extract. Alternatively, the nuclear extract may contain a fraction of p150 which is not part of the CAF-I complex and is inactive in chromatin assembly. An example of this has recently been described for the $cdc2^{human}$ protein kinase (the homologue of the $cdc2^{pombe}$ protein kinase), which is found in the cell in at least two forms: as an active kinase in a complex with other protein subunits or free of these subunits and inactive as a protein kinase (Draetta and Beach, 1988). Additional experiments using biochemical fractionation and immunoblotting analysis can be done to characterize the state of the individual subunits of CAF-I as they exist *in vivo*.

Recent studies on the mechanism of chromatin assembly during DNA replication have demonstrated that histones are assembled onto the DNA in a sequential manner (Smith and Stillman, 1991). In the first step, histones H3 and H4 are assembled onto the DNA during DNA replication in a CAF-1-dependent manner. Subsequently, histones H2A and H2B are loaded onto the DNA to complete the chromatin structure. These studies, and those described in this report, demonstrate that CAF-I plays an essential role in chromatin assembly and, therefore, may be a target for regulation during the cell cycle.

We have shown that both the p60 and the p150 subunits of CAF-I are phosphorylated. The function of this phosphorylation is unknown; however, several possibilities can be explored. Phosphorylation of CAF-I could be restricted to a specific time during the cell cycle associated with DNA replication as has recently been demonstrated for replication factor A, a multisubunit replication factor that is specifically phosphorylated during the S phase of the cell cycle (Din *et al.*, 1990). Phosphorylation in S phase could function in several ways: to localize proteins to specific regions of the nucleus, to regulate complex formation of a multisubunit protein, or to control the biochemical activity of a protein. With respect to this last point, we have previously shown that CAF-I binds histones *in vitro* (Smith and Stillman, 1989), a property which could be modulated by phosphorylation. Along these lines, it is interesting to note that the *Xenopus* chromatin assembly factor, nucleoplasmin, which binds histones *in vitro* (Laskey *et al.*, 1978; Earnshaw *et al.*, 1980) and *in vivo* (Dilworth *et al.*, 1987), becomes phosphorylated during development, coincident with an increase in chromatin assembly activity (Cotten *et al.*, 1986; Sealy *et al.*, 1986). Thus, it will be important to determine if the phosphorylation state of CAF-I effects its ability to bind histones and/or assemble chromatin *in vitro*.

In this report we describe the initial characterization of a panel of monoclonal antibodies directed against the human replication-dependent chromatin assembly factor, CAF-I. The ability to use these monoclonal antibodies across the wide range of techniques described here will permit an investigation of the function of CAF-I both *in vitro* and *in vivo*, with the ultimate goal of understanding how chromatin is assembled during DNA replication in eukaryotic cells.

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