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Postreplicative Chromatin Assembly by *Drosophila* and Human Chromatin Assembly Factor 1

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To study the relationship between DNA replication and chromatin assembly, we have purified a factor termed *Drosophila* chromatin assembly factor 1 (dCAF-1) to approximately 50% homogeneity from a nuclear extract derived from embryos. dCAF-1 appears to consist of four polypeptides with molecular masses of 180, 105, 75, and 55 kDa. dCAF-1 preferentially mediates chromatin assembly of newly replicated DNA relative to unreplicated DNA during T-antigen-dependent simian virus 40 DNA replication in vitro, as seen with human CAF-1. Analysis of the mechanism of DNA replication-coupled chromatin assembly revealed that both dCAF-1 and human CAF-1 mediate chromatin assembly preferentially with previously yet newly replicated DNA relative to unreplicated DNA. Moreover, the preferential assembly of the postreplicative DNA was observed at 30 min after inhibition of DNA replication by aphidicolin, but this effect slowly diminished until it was no longer apparent at 120 min after inhibition of replication. These findings suggest that the coupling between DNA replication and chromatin assembly may not necessarily involve a direct interaction between the replication and assembly factors at a replication fork.

Replication of the eukaryotic genome involves both the replication of DNA and the assembly of the newly synthesized DNA into chromatin (for reviews, see references 7, 11, 34, 35, and 36), and it is therefore important to study the relationship between DNA replication and chromatin assembly. The current data suggest that the assembly of chromatin in vivo begins immediately after DNA replication by a mechanism that leads to a random distribution of the newly synthesized and preexisting histones between the daughter DNA strands. In addition, it appears that nucleosome assembly occurs by the initial deposition of histones H3 and H4 followed by the incorporation of histones H2A and H2B. Structures that resemble bulk nucleosomes have been observed at replication forks by electron microscopy of chromatin obtained from early *Drosophila* embryos (21, 22). Yet in cultured cells, which divide much more slowly than nuclei in early *Drosophila* embryos, it has been found that the assembly of chromatin with the properties of bulk chromatin does not occur immediately after replication but instead requires about 5 to 30 min (7, 11, 27, 34, 37). It thus appears that although the process of chromatin assembly commences immediately after DNA replication, the steps in the assembly process may not necessarily occur concomitantly.

The analysis of chromatin assembly under nonreplicating conditions has led to the characterization of factors that mediate the reconstitution of nucleosomes in vitro. For instance, histone-binding proteins, such as nucleoplasmin, N1, and NAP-1, or nonphysiological polyanions, such as polyglutamate, can function as histone transfer vehicles that facilitate the random deposition of nucleosomes onto DNA in an ATP-independent manner (12, 17, 19, 23, 25, 31). In addition, crude extracts from *Drosophila*, *Xenopus*, or mammalian cells contain chromatin assembly factors that can mediate the ATP-facilitated and DNA replication-independent assembly of regularly spaced nucleosomal arrays (3, 4, 9, 13).

Biochemical studies of chromatin assembly during DNA replication have led to the identification, purification, and cloning of a factor termed chromatin assembly factor 1 (CAF-1) (15, 28–30, 32). CAF-1 was identified in studies of T-antigen-mediated simian virus 40 (SV40) DNA replication with a CAF-1-deficient, cytosolic replication extract derived from human 293 cells. In the absence of CAF-1, DNA replication with the 293 cell extract yields nonnucleosomal reaction products, whereas the addition of CAF-1 to the replication reactions results in the assembly of the newly replicated DNA into chromatin. Chromatin assembly with CAF-1 was found to occur preferentially on newly replicated DNA relative to the bulk unreplicated DNA in the reaction medium, and CAF-1-mediated chromatin assembly was not detected in the absence of DNA replication (30, 32).

In this report, we examine the CAF-1-mediated assembly of newly replicated DNA in the context of the SV40 DNA replication reaction. Thus far, CAF-1 has been identified and characterized only in human cells. However, earlier studies that we had performed on SV40 origin- and T-antigen-dependent DNA replication with factors derived from *Drosophila* embryos had suggested that there is a related CAF-1 activity in *Drosophila melanogaster* (14). Because of the potentially significant insight to be gained from the study of chromatin assembly in both mammals and *D. melanogaster*, we felt that it would be important to identify and to characterize the *Drosophila* CAF-1 activity. To this end, we describe in this report the partial purification and analysis of a factor from *Drosophila* embryos, which we term dCAF-1.

MATERIALS AND METHODS

Materials. SV40 large T antigen was purified by immunoaffinity chromatography from Sf9 cells infected with a recombinant baculovirus (18, 26). The human 293 cell cytosolic extract, which contains DNA replication factors but is deficient in CAF-1 activity, was prepared by the method of Stillman and Gluzman (33). The S-190 chromatin assembly extract, which contains dCAF-1, was

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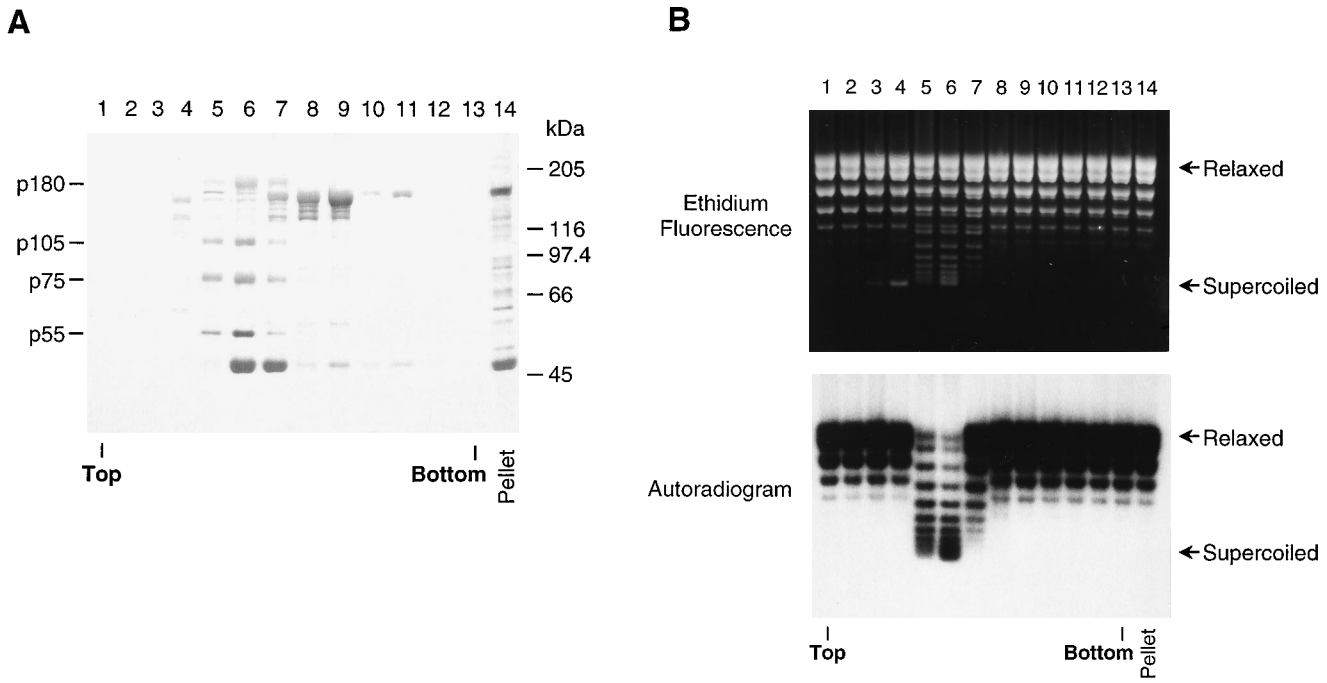

FIG. 1. Purification of dCAF-1 from *Drosophila* embryos. dCAF-1 was partially purified from a nuclear extract by ammonium sulfate fractionation and column chromatography as described in Materials and Methods. The dCAF-1 fraction was then subjected to 15 to 50% (vol/vol) glycerol gradient sedimentation, as shown in this figure and described in Materials and Methods. (A) Analysis of the protein composition of the glycerol gradient fractions by sodium dodecyl sulfate–7.5% (wt/vol) polyacrylamide gel electrophoresis. The proteins (300 μl of each fraction from a single ultracentrifuge tube) were precipitated with trichloroacetic acid, subjected to electrophoresis, and then visualized by staining with Coomassie brilliant blue R-250. Silver staining of the protein fractions reveals the same polypeptide composition (data not shown). The proteins shown in lane 14 were derived from the pellet at the bottom of the gradient. (B) Determination of CAF-1 activity by using the SV40 DNA replication-assembly assay. Replication reactions were performed under standard conditions with the same glycerol gradient fractions (9 μl) shown in panel A and the CAF-1-deficient human 293 cell extract (30, 32). The newly replicated DNA species were resolved by electrophoresis on a 1% (wt/vol) agarose gel and then detected by autoradiography of the dried gel (the replicated DNA was radiolabeled by the inclusion of [α-^{32}P]dATP in the reaction mixture). The upper panel shows the total DNA, as visualized by staining with ethidium bromide. The apparently supercoiled but nonradiolabeled DNA bands in lanes 3 and 4 of the ethidium bromide-stained agarose gel were not observed in several other similar sedimentation analyses of dCAF-1 and were therefore thought to be artifactual. The lower panel shows the newly replicated DNA, as visualized by autoradiography. The positions of relaxed and supercoiled DNA species are indicated.

prepared from *Drosophila* embryos (6, 13). The protein fraction containing partially purified CAF-1 from *Xenopus* eggs was the kind gift of H. Yan and J. Newport. This fraction was prepared by ammonium sulfate fractionation (37.5 to 52.5% saturation) of a crude extract derived from unfertilized *Xenopus* eggs (24). Purified recombinant human CAF-1 (hCAF-1) containing the p150 and p60 subunits was prepared as described previously (15). dCAF-1 was purified from a nuclear extract derived from embryos collected from 0 to 12 h after fertilization. dCAF-1 was purified by ammonium sulfate precipitation; successive chromatographic steps on SP-Sepharose FF (Pharmacia), Q-Sepharose FF (Pharmacia), heparin-Sepharose CL-2B (prepared from heparin and Sepharose CL-2B [Pharmacia]), SP-Sepharose FF (Pharmacia), Sephacryl S-400 HR (Pharmacia), and hydroxylapatite (Bio-Rad) resins; and glycerol gradient sedimentation. Through the hydroxylapatite column, there is an estimated 400-fold purification of dCAF-1 relative to the nuclear extract, with an estimated yield of 30%. The detailed methodology for the purification of dCAF-1 is available upon request.

Assays. CAF-1 activity was measured by carrying out SV40 DNA replication reactions (with the human 293 cell cytosolic extract, SV40 T antigen, pSV011 plasmid DNA, and [α-^{32}P]dATP) in either the presence or the absence of CAF-1 by using the procedure described previously (30, 32) and the specific conditions given by Kamakaka et al. (14). The CAF-1-dependent assembly of nucleosomes was then detected by the presence of negative supercoiling in the newly replicated DNA, which was detected by autoradiography. Because of the intrinsic inaccuracy in the determination of the low concentrations of protein in purified preparations of dCAF-1, the absolute amounts of dCAF-1 used in the assays should be viewed as rough estimates. For each preparation of dCAF-1, the amount of protein that was sufficient to mediate replication-associated chromatin assembly was determined empirically. The micrococcal nuclease digestion assay of the newly replicated DNA was performed by using the conditions described by Bulger and Kadonaga (6) for micrococcal nuclease digestion analysis of chromatin assembled with the S-190 extract except that unincorporated radiolabeled nucleotides were removed by precipitation of the DNA with ammonium acetate and isopropanol. Protein concentration was determined with the bicinchoninic acid protein assay (Pierce) by using bovine serum albumin as the reference. All experiments, including those for which data are not shown, were performed a minimum of two independent times, but more commonly four or more times, to ensure reproducibility of the data.

RESULTS

Identification, partial purification, and characterization of dCAF-1 from *Drosophila* embryos. In a previous study, we had observed a CAF-1-like activity in a crude extract derived from *Drosophila* embryos (14). In that work, it was shown that factors derived from *Drosophila* embryos were able to mediate SV40 origin- and T-antigen-dependent DNA replication in vitro. Moreover, the newly replicated DNA was supercoiled rather than relaxed, which suggested that the replicated DNA had been assembled into chromatin. Because CAF-1 has thus far been studied only in mammalian cells, it seemed likely that the analysis of a CAF-1-related activity from *D. melanogaster* would provide new information on CAF-1-mediated chromatin assembly. In addition, the ability to use *D. melanogaster* both for the characterization of the organism throughout development and for genetic analysis would enable experimental approaches and strategies for the study of CAF-1 that would not be readily available or technically feasible in mammals. Hence, we had sought to purify and to characterize the CAF-1 activity from *Drosophila* embryos.

By using conventional methodology (see Materials and Methods), dCAF-1 was purified to approximately 50% homogeneity from a crude nuclear extract (Fig. 1). CAF-1 activity was determined by carrying out T-antigen-mediated SV40

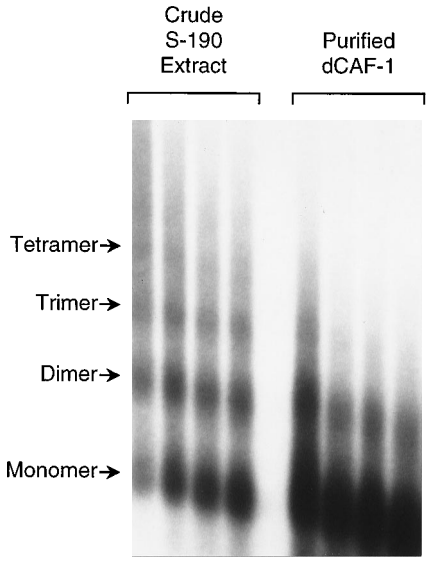

FIG. 2. Micrococcal nuclease digestion analysis of chromatin assembled onto newly synthesized DNA. SV40 DNA replication reactions were carried out under standard conditions at 30°C for 90 min in the presence of either crude *Drosophila* S-190 extract or purified dCAF-1, as indicated. The resulting chromatin was subjected to micrococcal nuclease digestion analysis (6), with various extents of digestion of the chromatin by micrococcal nuclease (reaction time of 10, 25, 40, or 60 min). An autoradiogram of the micrococcal nuclease digestion products is shown.

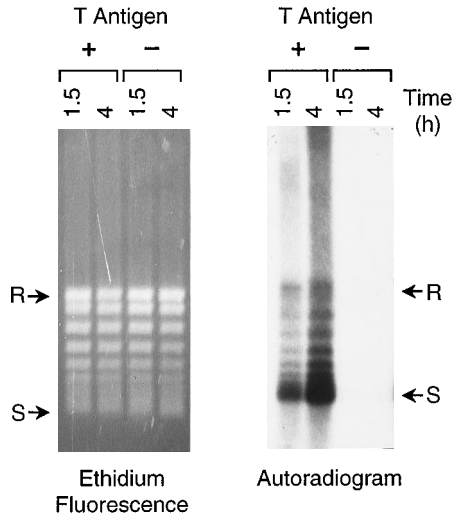

FIG. 3. Requirement for SV40 T antigen and DNA replication to observe chromatin assembly by dCAF-1 suggests that assembly is coupled to replication. SV40 DNA replication reactions were performed under standard conditions with purified dCAF-1 (80 ng of protein) in either the presence or the absence of purified SV40 large T antigen (2.2 μg of protein). The reactions were carried out for either 1.5 or 4 h, as indicated. The left panel shows the total DNA, as visualized by staining with ethidium bromide. The right panel shows the newly replicated DNA, as visualized by autoradiography. The positions of relaxed (R) and supercoiled (S) DNA species are indicated.

DNA replication reactions with a CAF-1-deficient replication extract derived from human 293 cells (30, 32). In this assay, the presence of CAF-1 was detected by the supercoiling of the newly replicated DNA. Four polypeptides with apparent molecular masses of 180, 105, 75, and 55 kDa copurified with dCAF-1 activity through several purification steps (the glycerol gradient sedimentation step is shown in Fig. 1), and we therefore suggest that these four polypeptides constitute dCAF-1. (Note, however, that the 180-kDa polypeptide does not perfectly correlate with dCAF-1 activity, as do the other three polypeptides. Nevertheless, on the basis of results of more than 15 independent preparations of dCAF-1, we believe that the 180-kDa subunit is a component of dCAF-1.) By comparison, hCAF-1 appears to consist of three polypeptides of 150, 60, and 50 kDa (15, 29, 30).

To confirm that the dCAF-1-induced supercoiling of the newly replicated DNA was due to the assembly of nucleosomes, we subjected the radiolabeled reaction products to micrococcal nuclease digestion analysis (Fig. 2). In this assay, the samples were partially digested with micrococcal nuclease, which cleaves both strands of DNA in the linker region between nucleosomal cores. The DNA fragments were deproteinized and then resolved by agarose gel electrophoresis. The DNA fragment ladder that was observed when replication reactions were performed in the presence of either a crude *Drosophila* extract or purified dCAF-1 revealed that the newly replicated DNA was assembled into chromatin, with a nucleosome repeat length of approximately 170 to 175 bp. It therefore appears that dCAF-1 mediates the assembly of nucleosomal arrays.

We also tested whether the radiolabeled DNA might have been generated by the nonspecific action of DNA polymerases, such as those involved in DNA repair. To this end, DNA replication-assembly reactions were performed in either the presence or the absence of SV40 T antigen (Fig. 3). In this experiment, DNA synthesis occurred only in the presence of T antigen, which indicates that it is unlikely that a DNA repair enzyme, such as DNA polymerase β, was responsible for the incorporation of the radiolabeled dATP into the template DNA. Moreover, comparison of the degree of DNA supercoiling of the bulk DNA (as detected by ethidium bromide staining) with that of the newly replicated DNA (as detected by autoradiography) revealed that dCAF-1-mediated assembly occurs preferentially with the newly replicated DNA relative to the bulk nonreplicated DNA, as seen with hCAF-1 (30, 32).

After the identification and purification of dCAF-1, we examined whether a CAF-1-related activity was present in *Xenopus laevis*, an organism from which egg or oocyte extracts are commonly prepared for the assembly of chromatin in vitro (9, 19, 24). We thus tested ammonium sulfate fractions (generously provided by H. Yan and J. Newport, University of California, San Diego) of a crude *Xenopus* egg extract (24) and identified an activity that mediated preferential assembly of newly replicated DNA in the SV40 DNA replication assay (Fig. 4). These findings suggest that CAF-1 is a factor that is conserved among *D. melanogaster*, *X. laevis*, and humans.

To determine the effect of the concentration of dCAF-1 on chromatin assembly, we carried out replication-assembly reactions in the presence of different amounts of dCAF-1 (Fig. 5A). As seen in Fig. 3 and 4, chromatin assembly by dCAF-1 occurs preferentially with the newly replicated DNA, but at the higher concentrations of dCAF-1, there was a considerable amount of supercoiling of the bulk DNA (Fig. 5A). (Under the conditions used in this study, typically 14 to 50% of the DNA molecules in the reaction products possess one newly synthesized DNA strand.) Because hCAF-1 has been previously shown to exhibit a distinct specificity for the assembly of newly replicated DNA relative to bulk DNA and was available as a purified, recombinant factor (30, 32), we then examined the ability of hCAF-1 to mediate nonreplicative chromatin assembly (Fig. 5B). In this experiment, we carried out a series of assembly reactions with

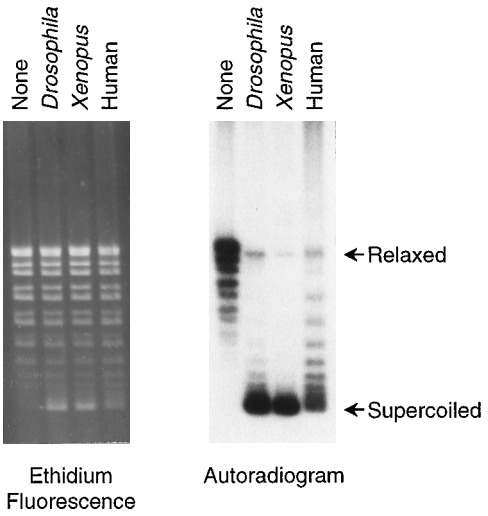

FIG. 4. DNA replication-associated chromatin assembly with CAF-1 activities derived from *Drosophila*, *Xenopus*, or human cells. SV40 DNA replication reactions were performed under standard conditions in either the absence or the presence of purified dCAF-1, a partially purified preparation of CAF-1 derived from unfertilized *Xenopus* eggs, or purified recombinant hCAF-1 (15). In the left panel, the total DNA was visualized by staining with ethidium bromide. In the right panel, the newly replicated DNA was visualized by autoradiography. The positions of relaxed and supercoiled DNA species are indicated.

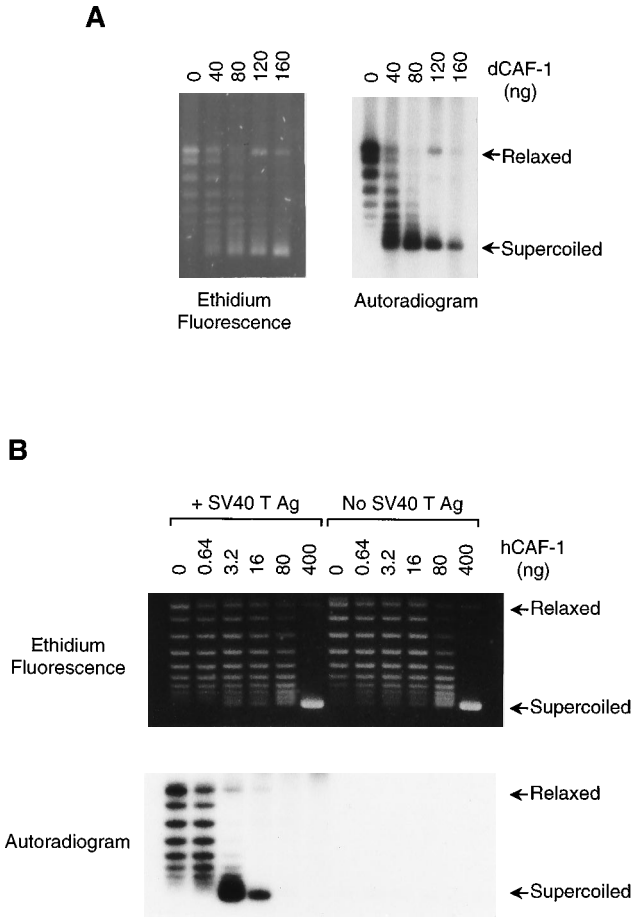

FIG. 5. Titration of CAF-1 activity reveals assembly of bulk, nonreplicated DNA. SV40 DNA replication-assembly reactions were performed under standard conditions with the indicated amounts of purified CAF-1. Where noted, the total DNA was visualized by staining with ethidium bromide, and the newly replicated DNA was visualized by autoradiography. The positions of relaxed and supercoiled DNA species are marked by arrows. (A) Chromatin assembly by dCAF-1. (B) Chromatin assembly by recombinant hCAF-1 (p150 and p60 subunits). In the right panel, SV40 large T antigen (Ag), which is required for initiation of replication, was omitted from the reactions.

increasing concentrations of purified recombinant hCAF-1 (p150 and p60 subunits) and found that hCAF-1 was also able to facilitate nonreplicative chromatin assembly, but only at concentrations that were between 25- and 100-fold higher than those required to observe chromatin assembly during DNA replication (Fig. 5B, left panel). (At the higher concentrations of hCAF-1, inhibition of DNA replication was observed, as reported previously [28, 30].) The hCAF-1-mediated, nonreplicative assembly was further shown to be independent of concurrent DNA replication by the omission of SV40 large T antigen in the reactions (Fig. 5B, right panel).

These data indicate that CAF-1, especially the purified recombinant hCAF-1, exhibits a strong preference for the assembly of newly replicated DNA relative to unreplicated DNA. The nonreplicative assembly that was observed, particularly that by the partially purified dCAF-1, might have been due to the following: (i) a weak, intrinsic ability of CAF-1 to function in the chromatin assembly process in the absence of DNA replication and/or (ii) a minor, contaminating assembly activity in the CAF-1 preparations. With regard to the former postulate, it is also possible that dCAF-1 possesses a more potent nonreplicative assembly activity than hCAF-1. On the other hand, with regard to the latter possibility, it should also be noted that the more highly purified recombinant hCAF-1 displayed a more stringent requirement for DNA replication than the partially purified dCAF-1. In the future, the cloning and subsequent characterization of purified, recombinant dCAF-1 should help to clarify these issues.

Chromatin assembly by dCAF-1 occurs preferentially with previously but newly replicated DNA relative to bulk, nonreplicated DNA. To gain a better understanding of the role of CAF-1 in replicative and nonreplicative chromatin assembly, we examined the ability of CAF-1 to assemble previously but newly synthesized DNA into chromatin in the context of the SV40 replication reaction. In the first set of experiments, we carried out DNA replication reactions to which either dCAF-1 or buffer (as a control) was added to the reaction medium at various time points after the initiation of replication (Fig. 6). In the course of a standard 80-min replication-assembly reaction, there was a significant amount of dCAF-1-dependent chromatin assembly of the newly replicated DNA (Fig. 6; compare lanes 6 and 11). By comparison, when dCAF-1 was added to the replication reaction at 64 min after initiation and the mixture was incubated for an additional 16 min (to a total reaction time of 80 min), most of the newly replicated DNA was also highly supercoiled (Fig. 6, autoradiogram, lane 10; compare with the control reaction shown in lane 15, in which buffer only was added instead of dCAF-1). Because the amount of relaxed, circular, newly replicated DNA that was synthesized in 64 min of total reaction time in the absence of dCAF-1 (Fig. 6, autoradiogram, lane 5) was significantly higher than the corresponding amount of relaxed, circular, newly replicated DNA that remained after the addition of dCAF-1 (after 64 min of reaction time in the absence of dCAF-1, followed by an additional 16 min in the presence of dCAF-1) (Fig. 6, autoradiogram, lane 10), we conclude that the relaxed, circular, newly replicated DNA that was synthesized during the first 64 min in the absence of dCAF-1 was postreplicatively assembled into

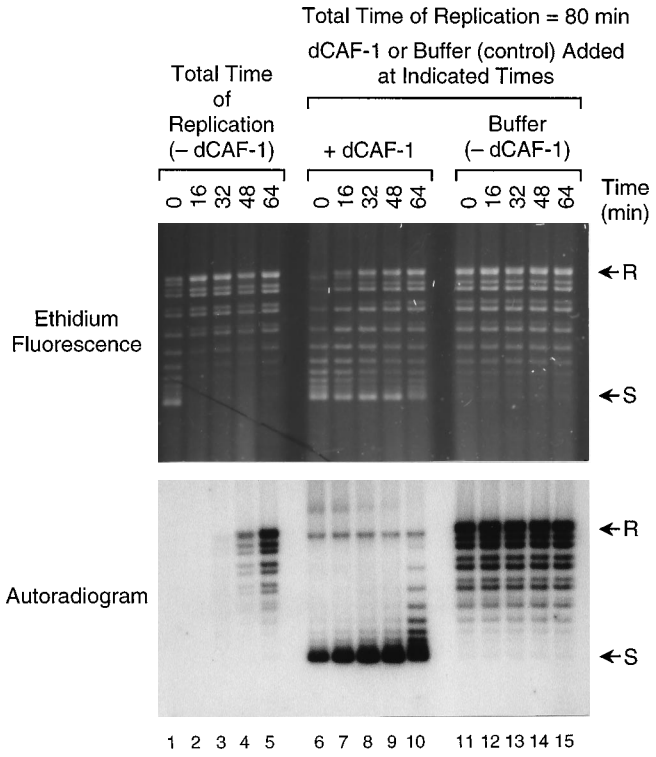

FIG. 6. Assembly of chromatin by dCAF-1 exhibits a preference, but not a requirement, for newly synthesized DNA relative to bulk, nonreplicated DNA. SV40 DNA replication reactions were carried out under standard conditions. In lanes 1 to 5, the total reaction time was varied from 0 to 64 min, as shown. In lanes 6 to 15, the total reaction time was 80 min, and either dCAF-1 (~150 ng of protein purified through the hydroxylapatite column) or buffer (as a control) was added at the indicated times after initiation of the reaction. The upper panel shows the total DNA, as visualized by staining with ethidium bromide. The lower panel shows the newly replicated DNA, as visualized by autoradiography. The positions of relaxed (R) and supercoiled (S) DNA species are indicated. In lane 1, a significant fraction of the bulk DNA is supercoiled because the negatively supercoiled plasmid DNA that was added to the reaction medium had not been relaxed by the endogenous topoisomerases in the course of the short reaction time (~5 s≅ 0 min).

chromatin by dCAF-1. Moreover, comparison of the extent of chromatin assembly of the bulk (and mainly unreplicated) DNA (Fig. 6, ethidium fluorescence) with that observed with the newly replicated DNA (Fig. 6, lane 10; autoradiogram, lane 10) revealed that the previously but newly replicated DNA was preferentially assembled into chromatin relative to the bulk DNA.

We then tested the ability of dCAF-1 to mediate the assembly of newly replicated DNA into chromatin under conditions wherein DNA replication was inhibited by the presence of aphidicolin (Fig. 7A). In this experiment, a single, large-scale (10 times the normal size) SV40 replication reaction was carried out in the absence of dCAF-1 for 90 min, after which the replication reaction was inhibited by the addition of aphidicolin (quantitative analysis of the newly replicated DNA in Fig. 7A, lanes 5 to 14, had indicated that no additional DNA synthesis had occurred after the addition of aphidicolin; data not shown). The reaction mixture was divided into two equal portions to which either dCAF-1 (lanes 5 to 9) or buffer only (as a control; lanes 10 to 14) was added, and then aliquots (one reaction equivalent each) were removed at the indicated times (after the addition of dCAF-1 or buffer) to observe dCAF-1-mediated assembly of the previously yet newly replicated DNA. As seen in Fig. 7A, dCAF-1 was able to facilitate chromatin assembly in the apparent absence of DNA synthesis. Furthermore, consistent with the results shown earlier in Fig. 6, this experiment revealed a distinct preference for the assembly of the postreplicative DNA relative to the bulk DNA (Fig. 7A, lanes 7 to 9). It is important to note that at the concentration of dCAF-1 used in these reactions, only a small extent of nonreplicative chromatin assembly was observed when aphidicolin was added at the beginning of the 90-min reaction period (Fig. 7A, upper panel; compare lanes 1 and 2). It therefore appears that the supercoiled DNA seen by ethidium fluorescence in the presence of dCAF-1 (Fig. 7A, upper panel; compare lane 1, in the absence of replication, with lanes 3, 7, 8, and 9) is predominantly newly replicated DNA, which typically constitutes about 14 to 50% of the total DNA after replication under these reaction conditions. Thus, these data further support the conclusion that there is a preference for the assembly of postreplicative DNA relative to the unreplicated DNA under these conditions.

We also carried out parallel studies of DNA replication and chromatin assembly with hCAF-1 (Fig. 7B). The properties of hCAF-1 were nearly identical to those of dCAF-1, although the rate of postreplicative chromatin assembly with hCAF-1 (Fig. 7B) was consistently slower than that with dCAF-1 (Fig. 7A). Notwithstanding these differences, the results with both dCAF-1 and hCAF-1 support the model that DNA replication and chromatin assembly by CAF-1 are coupled processes, but that concurrent DNA replication is not obligatorily required for the assembly of chromatin by CAF-1.

Lastly, we examined whether the previously yet newly replicated DNA can stably remain a preferential substrate (relative to unreplicated DNA) for CAF-1-mediated chromatin assembly (Fig. 8). To this end, we performed a single, large-scale SV40 replication reaction in the absence of dCAF-1 for 90 min, after which further replication was inhibited by the addition of aphidicolin, as in Fig. 7. Then, at various times (1, 30, 60, and 120 min) after the addition of aphidicolin, aliquots (one reaction equivalent each) were transferred to new tubes containing either dCAF-1 (Fig. 8, lanes 5 to 8) or buffer only (as a control; Fig. 8, lanes 9 to 12), and chromatin assembly was allowed to proceed for an additional 60 min. As shown in Fig. 8, the preferential ability of CAF-1 to mediate assembly of the postreplicated DNA was still observed, though to a slightly lesser extent, upon 30 min of incubation of the newly replicated DNA in reaction medium. Upon further incubation, however, the preferential assembly of the newly replicated DNA was no longer seen (Fig. 8, lane 8).

These results, combined with those of Fig. 7, suggest that a potentiated state of the newly replicated DNA, such as an intrinsic property of the DNA itself or the association of a factor with the DNA, renders the newly replicated DNA a better substrate for CAF-1-mediated chromatin assembly than unreplicated DNA. Moreover, this state of the newly replicated DNA is stable for about 30 min after inhibition of replication, after which the preferential assembly of the postreplicative DNA is diminished. The loss of the preferential assembly may be due to loss of this special property of the newly replicated DNA and/or the loss of the activity of other assembly factors in the replication-assembly extract (i.e., factors other than CAF-1, which are also needed for chromatin assembly) during the extended incubation period. In the future, it will be important to determine the molecular basis for the preferential assembly of newly replicated DNA relative to unreplicated DNA.

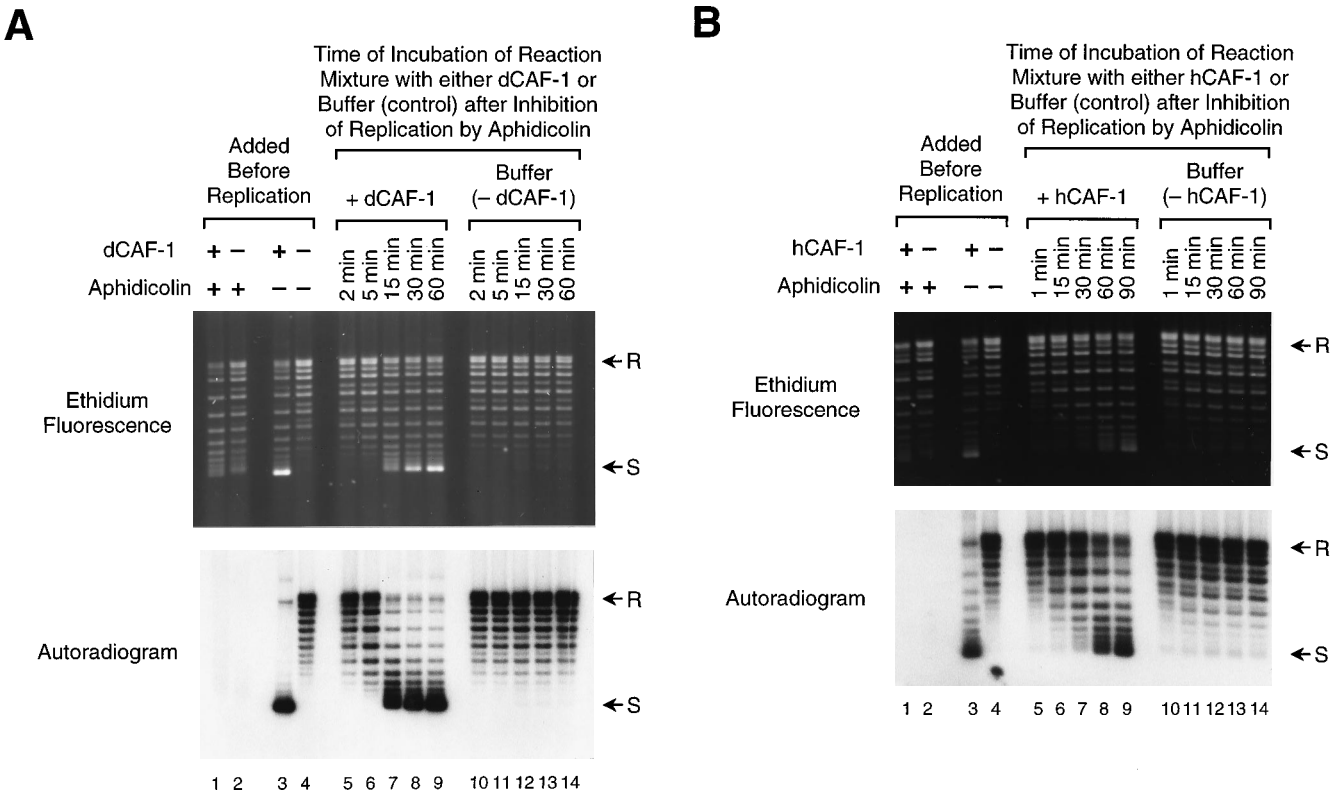

FIG. 7. Postreplicative assembly of chromatin by dCAF-1 and hCAF-1. SV40 DNA replication reactions were carried out under standard conditions. In lanes 1 to 4, the total reaction time was 90 min; where indicated, aphidicolin (5 μg/ml, final concentration) and/or CAF-1 were included in the reactions throughout the entire reaction period. In lanes 5 through 14, a single, large-scale reaction (10 times the size of a normal reaction) was performed in the absence of CAF-1 for 90 min. At 90 min, aphidicolin was added to the reaction medium to a final concentration of 5 μg/ml. Then, the sample was divided into two equal portions to which either CAF-1 (lanes 5 to 9) or buffer (as a control; lanes 10 to 14) was added. At the indicated times after the addition of aphidicolin (and either CAF-1 or buffer, as a control), aliquots (one reaction equivalent each) were removed and deproteinized to give the samples shown in lanes 5 to 14. The upper panel shows the total DNA, as visualized by staining with ethidium bromide. The lower panel shows the newly replicated DNA, as visualized by autoradiography. The positions of relaxed (R) and supercoiled (S) DNA species are indicated. (A) Chromatin assembly by dCAF-1 (~150 ng of protein purified through the hydroxylapatite column). (B) Chromatin assembly by hCAF-1 (~4 ng of purified, native hCAF-1).

DISCUSSION

We have identified and purified dCAF-1 as a factor that participates in DNA replication-coupled chromatin assembly. We have further found that both dCAF-1 and hCAF-1 can mediate the preferential assembly of newly replicated DNA relative to unreplicated DNA in the absence of ongoing DNA replication. The ability of dCAF-1 to function postreplicatively may be related to the results of a study of nonreplicative chromatin assembly (5), in which we had fractionated the *Drosophila* S-190 chromatin assembly extract and identified a dCAF-1-containing fraction that mediates the assembly of regularly spaced nucleosomal arrays in conjunction with a 56 kDa core histone-binding protein, core histones, DNA, and ATP. (It should be noted, however, that in that study, dCAF-1 was not unambiguously identified as the active component [or one of the active components] of the dCAF-1-containing fraction.) Moreover, though this work has focused primarily on dCAF-1 and hCAF-1, we have also shown that CAF-1 is present in unfertilized *Xenopus* eggs (Fig. 4). Hence, the DNA replication-associated (1, 2) as well as the ATP-facilitated and DNA replication-independent assembly of chromatin that has been observed in extracts derived from *Xenopus* eggs or oocytes (9, 24) might also require CAF-1.

Nature of the coupling of DNA replication and chromatin assembly. The observation that dCAF-1 preferentially mediates the postreplicative assembly of newly replicated DNA relative to bulk (and mainly unreplicated) DNA leads to the suggestion that the coupling between DNA replication and dCAF-1-mediated chromatin assembly can be indirect. It is, moreover, difficult to envisage a continuous mechanical linkage between synthesis of the lagging-strand DNA and chromatin assembly because RNA priming as well as maturation and ligation of the Okazaki fragments are interleaved with DNA synthesis at the lagging strand. This notion is consistent with the observation that chromatin assembly was found to occur in the apparent absence of DNA replication in vivo in *Saccharomyces cerevisiae* (16). In those experiments, histone H4 synthesis was blocked in cells that were synchronized in G_1, which had subsequently resulted in cells with nucleosome-deficient chromosomes that were arrested in G_2. Then, reinduction of H4 synthesis in the G_2-arrested cells had led to nucleosome assembly on nonreplicating DNA. This conclusion should, however, be interpreted with some caution because the normal cellular physiology was affected by the inhibition of H4 synthesis.

Postreplicative assembly of chromatin has also been reported to be mediated by a possible CAF-1-like activity in a nuclear extract derived from Manca (human Burkitt lymphoma) cultured cells (8). In those studies, however, it was not certain whether the postreplicative assembly was due to CAF-1 or to some other component in the reaction medium. In addition, another study of DNA replication and chromatin assem-

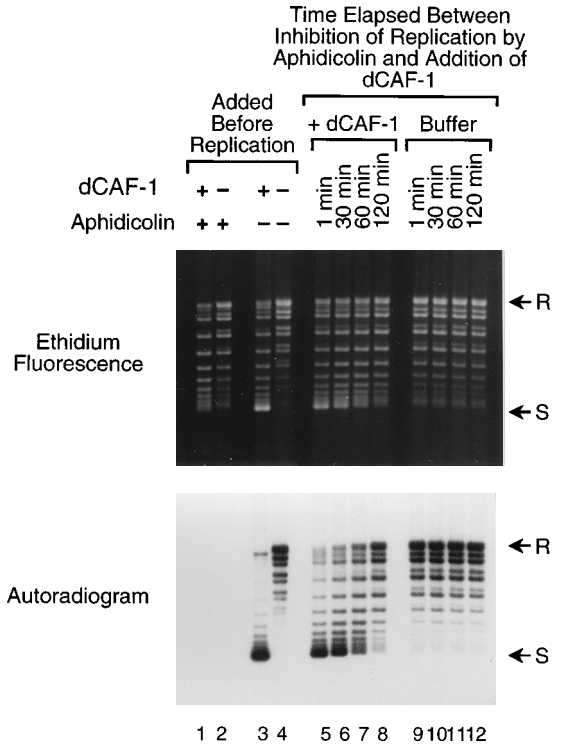

FIG. 8. Stability of preferentially assembled, postreplicative DNA. SV40 DNA replication reactions were carried out under standard conditions. In lanes 1 to 4, the total reaction time was 90 min; where indicated, aphidicolin (5 μg/ml, final concentration) and/or dCAF-1 (~150 ng of protein purified through the hydroxylapatite column) were included in the reactions throughout the entire reaction period. In lanes 5 through 12, a single, large-scale reaction (10 times the size of a normal reaction) was performed in the absence of CAF-1 for 90 min. At 90 min, aphidicolin was added to the reaction medium to a final concentration of 5 μg/ml. At the indicated times after the addition of aphidicolin, aliquots (one reaction equivalent each) were transferred to new tubes and added to either dCAF-1 (lanes 5 to 8) or buffer (as a control; lanes 9 to 12). After addition to dCAF-1 or buffer, the aliquots were incubated at 30°C for 60 min before deproteinization to give the samples shown in lanes 5 to 12. The upper panel shows the total DNA, as visualized by staining with ethidium bromide. The lower panel shows the newly replicated DNA, as visualized by autoradiography. The positions of relaxed (R) and supercoiled (S) DNA species are indicated.

bly had led to the suggestion that nucleosome assembly in mammalian cell extracts was not dependent on DNA replication (10). It was not evident, however, whether the chromatin assembly process that had been observed in that study was similar to that described in this work and other studies of CAF-1, as some mammalian cell extracts have also been found to be competent for DNA replication-independent assembly of bulk DNA (see, for example, reference 3). It is possible, as also suggested elsewhere (20), that the extracts used in that study (10) contained factors that could mediate both CAF-1-coupled as well as CAF-1-independent chromatin assembly.

How does dCAF-1 distinguish between previously replicated DNA and unreplicated DNA? It might be thought, for example, that a subnucleosomal particle, such as an H3-H4 tetramer, is deposited onto the DNA template during replication and then further assembled postreplicatively into nucleosomes by CAF-1. This possibility is unlikely because it has been shown (28) that CAF-1 is required for the deposition of H3 and H4 onto the DNA template during replication to yield an assembly intermediate that can be postreplicatively converted into chromatin upon the addition of histones H2A and H2B. Another possible explanation for the discrimination between previously replicated and bulk nonreplicated DNA is that some aspect of the structure or topology of the newly replicated DNA is different from that of the nonreplicated DNA. Lastly, it should also be considered that other non-histone protein factors that facilitate nucleosome assembly might be associated with the newly replicated DNA.

Comparison of the properties of dCAF-1 and hCAF-1. Throughout this work, we have found that the chromatin assembly activities of dCAF-1 are similar to those of hCAF-1. In addition, both dCAF-1 and hCAF-1 exhibit much larger apparent molecular masses by gel filtration chromatography (~500 to 700 kDa) than by glycerol gradient sedimentation (~120 kDa) (reference 30 and data not shown). The major differences in the properties of dCAF and hCAF-1 were seen in the nonreplicative and postreplicative assembly reactions. For instance, as shown in Fig. 5, the preference of hCAF-1 for assembly of newly replicated DNA relative to unreplicated DNA was more distinct than that of dCAF-1. Also, as depicted in Fig. 7, the rate of postreplicative assembly by hCAF-1 was slower than that by dCAF-1. It thus appears that dCAF-1 has a greater nonreplicative assembly activity than hCAF-1, which may be due to an intrinsic function of the *Drosophila* factor, a special property of dCAF-1 from early *Drosophila* embryos (in which there are very high levels of DNA replication and chromatin assembly), or the action of another protein present in the partially purified dCAF-1 fraction. Notwithstanding these differences, the fundamental similarities in the properties of dCAF-1 and hCAF-1 suggest that they are homologous factors.

Summary and perspectives. CAF-1 is an activity that is present in *Drosophila*, *Xenopus*, and human cells. Both dCAF-1 and hCAF-1 exhibit a preference for the assembly of chromatin onto newly replicated DNA. Moreover, studies of the assembly of previously yet newly replicated DNA suggest that newly replicative DNA is a preferential substrate for CAF-1-mediated assembly relative to unreplicated DNA even in the absence of concomitant DNA replication. It will be interesting to investigate further the molecular basis of the coupling of DNA replication and chromatin assembly. In addition, with dCAF-1, we hope, in the future, to study the function of the factor from the perspective of the growth and development of the entire organism.

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