

# Assembly of regularly spaced nucleosome arrays by *Drosophila* chromatin assembly factor 1 and a 56-kDa histone-binding protein

(nucleosome assembly)

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**ABSTRACT** To ascertain the mechanism by which nucleosomes are assembled by factors derived from *Drosophila* embryos, two proteins termed *Drosophila* chromatin assembly factors (CAFs) 1 and 4 (dCAF-1 and dCAF-4) were fractionated and purified from a *Drosophila* embryo extract. The assembly of chromatin by dCAF-1, dCAF-4, purified histones, ATP, and DNA is a process that generates regularly spaced nucleosomal arrays with a repeat length that resembles that of bulk native *Drosophila* chromatin and is not obligatorily coupled to DNA replication. The assembly of chromatin by dCAF-1 and dCAF-4 is nearly complete within 10 min. The dCAF-1 activity copurified with the *Drosophila* version of chromatin assembly factor-1 (CAF-1), a factor that has been found to be required for the assembly of chromatin during large tumor (T) antigen-mediated, simian virus 40 (SV40) origin-dependent DNA replication. The dCAF-4 activity copurified with a 56-kDa core-histone-binding protein that was purified to >90% homogeneity.

DNA (and hence, chromatin) is a central component of many cellular processes; thus, the mechanism by which chromatin is assembled is relevant to a broad range of biological phenomena (for reviews, see refs. 1–5). The analysis of nucleosome assembly *in vivo* has revealed that assembly commences immediately after DNA replication but may not be obligatorily coupled to DNA replication. On the other hand, biochemical studies of chromatin assembly have led to the identification of core-histone-binding factors, which include nucleoplasmin, N1/N2, nucleosome assembly protein 1 (NAP-1), and RNA, that can mediate the DNA-replication- and ATP-independent deposition of nucleosomes that do not exhibit regular nucleosomal spacing (see, for example, refs. 6–11). In addition, a factor termed chromatin assembly factor 1 (CAF-1) has been found to be required for nucleosome assembly during large tumor (T) antigen-mediated, simian virus 40 (SV40) origin-dependent DNA replication (12–17). CAF-1 has been characterized by using a biochemical complementation assay with a CAF-1-deficient DNA replication extract, and purified human CAF-1 appears to exhibit a preference for the assembly of nucleosomes onto newly synthesized DNA relative to bulk nonreplicated DNA (refs. 12–14; but also see refs. 18 and 19). Up to the present, however, the factors that function along with CAF-1 to assemble chromatin have not been identified.

It also has been demonstrated that factors present in crude extracts of either *Xenopus* or *Drosophila* can mediate the DNA-replication-independent assembly of nucleosomes that are approximately regular in spacing (which we will henceforth refer to as “regularly spaced nucleosomes”) in the presence of ATP (see, for example, refs. 20–23). Biochemical characterization of *Xenopus* extracts (derived from either oocytes or unfertilized eggs) has revealed that the core-histone-binding factors, nucleoplasmin and N1/N2, which may perhaps be

thought of as histone chaperones, are involved in the assembly of chromatin (6–8, 24, 25). Neither CAF-1 nor NAP-1 was identified as activities that were required for nucleosome assembly with the fractionated *Xenopus* extracts. Yet chromatin that is reconstituted with purified nucleoplasmin and/or N1/N2 does not possess regularly spaced nucleosomal arrays, and it has been postulated that there may be additional factors that are required for the generation of the periodic spacing of nucleosomes (24–28). Thus, to date, a variety of histone-binding factors that randomly deposit nucleosomes have been characterized, but the set of factors, whether in purified or partially purified fractions, that is sufficient for the assembly of regularly spaced nucleosome arrays has not yet been fractionated and identified.

In this work, we have sought to analyze the mechanism of assembly of regularly spaced nucleosomal arrays by fractionation and purification of factors from a crude extract, termed the S-190, that is derived from predominantly postblastoderm *Drosophila* embryos (22, 23). These studies have led to the identification of two activities, termed *Drosophila* CAFs 1 and 4 (dCAF-1 and dCAF-4), that are required in addition to core histones, ATP, and DNA for the efficient assembly of periodic nucleosomal arrays.

## MATERIALS AND METHODS

**Chromatin Assembly.** Chromatin assembly reactions were performed as described (22, 23) with slight modification. Circular plasmid DNA was relaxed by incubation with purified recombinant *Drosophila* topoisomerase I for 30 min under conditions identical to those described for the native enzyme (29, 30). A standard chromatin assembly reaction (75- $\mu$ l final volume) was carried out as follows: relaxed circular DNA (600 ng; with topoisomerase I) was combined with dCAF-1 [24  $\mu$ g of protein from the 0.25–0.5 M potassium phosphate fraction eluted from the hydroxylapatite column in Fig. 1A (except as indicated in Figs. 3B and 4B)], dCAF-4 [40  $\mu$ g of protein from the 0.3–0.4 M NaCl fraction eluted from the Q-Sepharose column in Fig. 1A (except as indicated in Fig. 4B)], purified *Drosophila* core histones (500 ng), 3 mM ATP (and ATP regeneration system comprising 30 mM phosphocreatine and 1  $\mu$ g of creatine phosphokinase per ml), and bovine serum albumin as a carrier protein (150 ng). Where indicated, purified *Drosophila* histone H1 was also included. All reactions, except as indicated in Fig. 1E, were incubated at 27°C for 4 hr. In reactions containing 3 mM ATP, MgCl<sub>2</sub> was added to a final concentration of 5 mM, while in reactions that did not contain ATP, MgCl<sub>2</sub> was added to final concentration of 2 mM. It is also pertinent to note that chromatin assembly with the purified factors was not sensitive to the Mg<sup>2+</sup> concentration, as the extent of chromatin assembly was not affected by

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Abbreviations: CAF-1, chromatin assembly factor I; NAP-1, nucleosome assembly protein I; T antigen, large tumor antigen; dCAF-1 and dCAF-4, *Drosophila* CAFs 1 and 4; SV40, simian virus 40.

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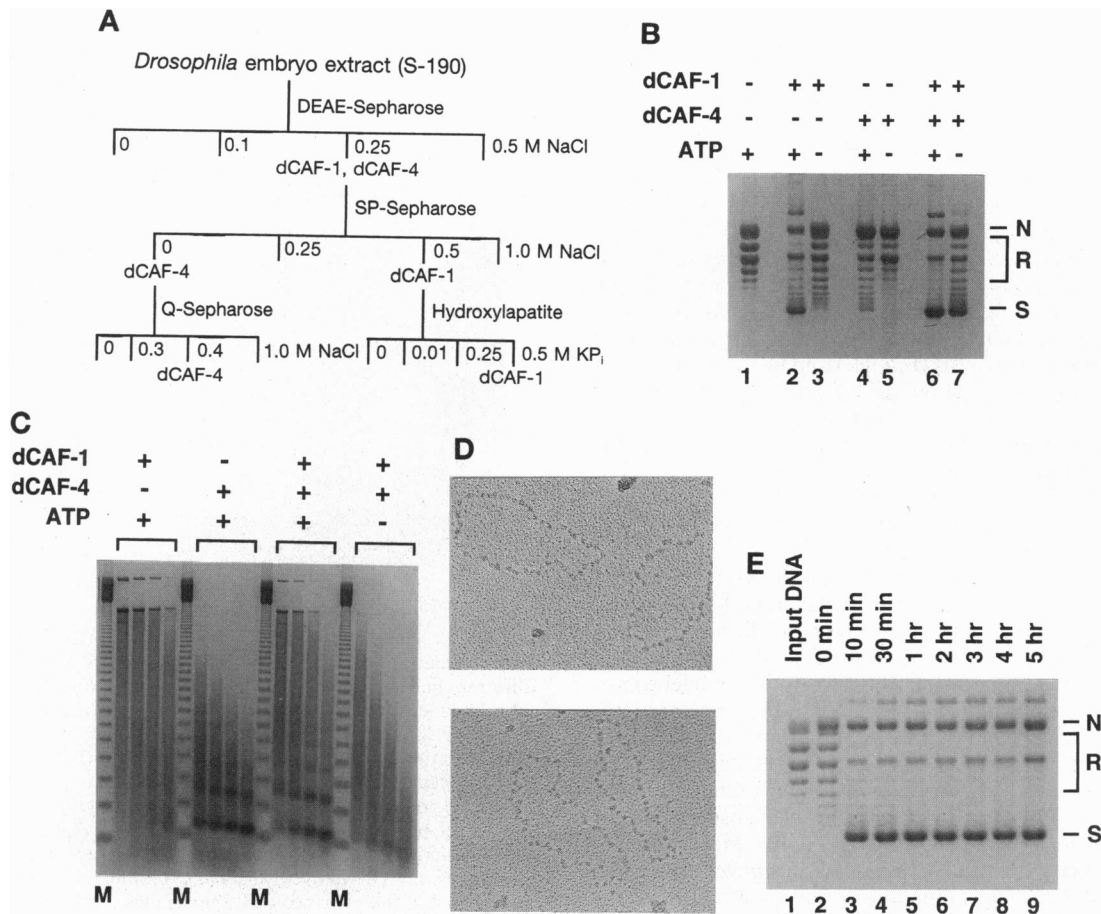


FIG. 1. Assembly of chromatin by partially purified dCAF-1 and dCAF-4 yields regularly spaced nucleosomal arrays. (A) Scheme for the fractionation of the *Drosophila* S-190 extract. (B) DNA supercoiling analysis. Chromatin assembly reactions were carried out with relaxed circular DNA (3.25-kbp plasmid), and dCAF-1, dCAF-4, and ATP were included, as indicated. After 4 hr, the samples were deproteinized. The resulting DNA was subjected to 1% agarose gel electrophoresis and then visualized by staining with ethidium bromide. The positions of relaxed (R), supercoiled (S), and nicked circular (N) forms of DNA are denoted. (C) Micrococcal nuclease digestion analysis. For each reaction condition, the different lanes represent increasing concentrations (from left to right) of micrococcal nuclease used to digest the chromatin as described in text. Chromatin assembly reactions were carried out in either the presence or the absence of the indicated factors. The samples were partially digested with various concentrations of micrococcal nuclease and then deproteinized. The resulting DNA fragments were subjected to agarose gel electrophoresis and visualized by staining with ethidium bromide. The molecular mass marker (lanes M) is the 123-bp ladder (GIBCO/BRL). (D) Electron microscopy. Chromatin was assembled and then purified by 30–50% sucrose gradient sedimentation, desalted with Sepharose CL-4B (Pharmacia Biotech), and analyzed by electron microscopy. (E) Time course of assembly by dCAF-1 and dCAF-4, as measured by the DNA supercoiling assay. Lanes: 1, relaxed circular DNA used in the assembly reaction; 2–9, aliquots from a chromatin assembly reaction that were removed and deproteinized at the indicated times.

variation of the  $Mg^{2+}$  concentration from 1.3–7 mM (unpublished data). Each lane in the DNA supercoiling and micrococcal nuclease assays represents the DNA derived from one unit reaction.

**Assays.** In the micrococcal nuclease assays, four 75- $\mu$ l aliquots were taken from a single larger scale reaction mixture. To each aliquot, 0.1 M  $CaCl_2$  was added to 3 mM, and then micrococcal nuclease was added to 0.11, 0.23, 0.45, or 0.90 unit/ml. Digestion was allowed to proceed for 10 min, and the reaction was stopped by addition of EDTA (pH 8.0) to 10 mM and of RNase A to 20 ng/ $\mu$ l. In the DNA supercoiling assays, the reaction products sometimes appear as a single band at the bottom of the gel. This band represents a distribution of highly supercoiled DNAs that are not resolved under the electrophoretic conditions. For each of the reactions shown, we have also resolved these supercoiled species by electrophoresis in the presence of chloroquine (data not shown), and those results confirmed our interpretation of the agarose gels shown in the figures. The T antigen- and SV40 origin-dependent SV40 DNA replication reactions for replicative CAF-1 activity were carried out as described (12, 13). Reactions under all conditions, whether shown or indicated as unpublished data,

were performed a minimum of two times (but usually three or more times) to ensure reproducibility of the data. Supplementary material that provides a detailed description of the fractionation and purification of the assembly factors is available upon request.

## RESULTS AND DISCUSSION

**Assembly of Chromatin by Partially Purified dCAF-1 and dCAF-4.** The initial fractionation and partial purification of chromatin assembly factors dCAF-1 and dCAF-4 are depicted in Fig. 1A. To monitor the proper assembly of chromatin and to minimize potential artifacts that could have arisen during the fractionation, we employed three assays—DNA supercoiling (31, 32), micrococcal nuclease digestion (33), and electron microscopy (34). At this early stage of the fractionation, the dCAF-1 and dCAF-4 activities were not yet completely resolved, and there was some contaminating RNA in the dCAF-1 fraction (data not shown), which had contributed to ATP-independent nucleosome deposition (11). Nevertheless, the assembly of chromatin was found to be most efficient in the presence of dCAF-1, dCAF-4, and ATP (Fig. 1 B and C).

Electron microscopy of the chromatin revealed that the templates were fully assembled (Fig. 1D). Furthermore, quantitative analysis of the DNA supercoiling by two-dimensional agarose gel electrophoresis revealed an average of 21 nucleosomes per 3.25-kbp template in the absence of histone H1, which corresponds to 1 nucleosome per 155 bp of DNA (data not shown). Further purification of the factors, as shown below, subsequently clarified the requirement for dCAF-1, dCAF-4, and ATP for efficient chromatin assembly.

**Rate of Chromatin Assembly by dCAF-1 and dCAF-4.** We have also investigated the rate of chromatin assembly by dCAF-1 and dCAF-4. These experiments revealed that chromatin assembly was nearly complete after 10 min, as measured by DNA supercoiling analysis (Fig. 1E) and by the micrococcal nuclease digestion assay (unpublished data). By comparison, with a *Xenopus* S-150 oocyte extract (35), 1–2 hr of reaction time is required to achieve the same extent of assembly that is observed in 10 min with dCAF-1 and dCAF-4. Under these conditions, assembly is nearly complete within 10 min, which is comparable to the rate of chromatin assembly that has been observed *in vivo* (36–39).

**Nucleosome Repeat Length of Chromatin Assembled with dCAF-1 and dCAF-4.** We then examined factors that affect the nucleosome repeat length. In the absence of histone H1, the repeat length varied with the monovalent salt concentration in the reaction medium as follows: 155 bp (21 nucleosomes per 3.25-kbp plasmid) at 7 mM KCl; 162 bp (20 nucleosomes per plasmid) at 57 mM KCl; and 180 bp (18 nucleosomes per plasmid) at 107 mM KCl (Fig. 2A). It has been shown previously that the incorporation of histone H1 into chromatin during assembly *in vitro* increases the nucleosome repeat length (see, for example, refs. 21, 22, and 40). Moreover, the apparent absence of H1 in the yeast *Saccharomyces cerevisiae* corresponds with the short (165 bp) nucleosome repeat length in that organism. We have therefore examined the effect of histone H1 upon nucleosomal spacing upon assembly by dCAF-1 and dCAF-4 and found that the repeat length of the H1-containing chromatin (190 bp; 17 nucleosomes per 3.25-kbp plasmid) resembled that of native H1-containing *Drosophila* chromatin (Fig. 2B) and was not significantly affected by variation of the KCl concentration in the reaction medium from 7 to 107 mM (unpublished data).

These results indicate that the assembly of histone H1-containing chromatin by dCAF-1 and dCAF-4 yields a nucleosomal array with a repeat length that resembles that of bulk native (H1-containing) chromatin from *Drosophila* embryos. In the absence of H1, the repeat length increases with the concentration of monovalent salt in the reaction medium. It is possible that both the salt and the histone H1, which has lysine-rich N- and C-terminal tails, act similarly as charged species to affect the repeat length, which may be a function of histone–histone and/or histone–DNA interactions.

**The dCAF-1 Fraction Contains the *Drosophila* Version of Human CAF-1.** Next, we sought to characterize the active components of dCAF-1 and dCAF-4, and to this end we have found that the dCAF-1 fraction contains a functional homologue of human CAF-1. The chromatographic column fractions shown in Fig. 1A were tested for CAF-1 activity by carrying out T antigen- and SV40 origin-dependent DNA replication reactions with a CAF-1-deficient replication extract (12, 13) (Fig. 3A). This experiment revealed that the nonreplicative dCAF-1 activity (as shown in Fig. 1A) copurifies with the replicative CAF-1 activity through three chromatographic purification steps. In separate studies, we have also purified the *Drosophila* version of CAF-1 to  $\approx 30\%$  homogeneity by using the SV40 replication assay (R.T.K., M.B., P. D. Kaufman, B. Stillman, and J.T.K., unpublished data), and this highly purified *Drosophila* CAF-1 substitutes for the dCAF-1 fraction in the nonreplicative assembly reaction (Fig. 3B). It is therefore likely that an active component in the

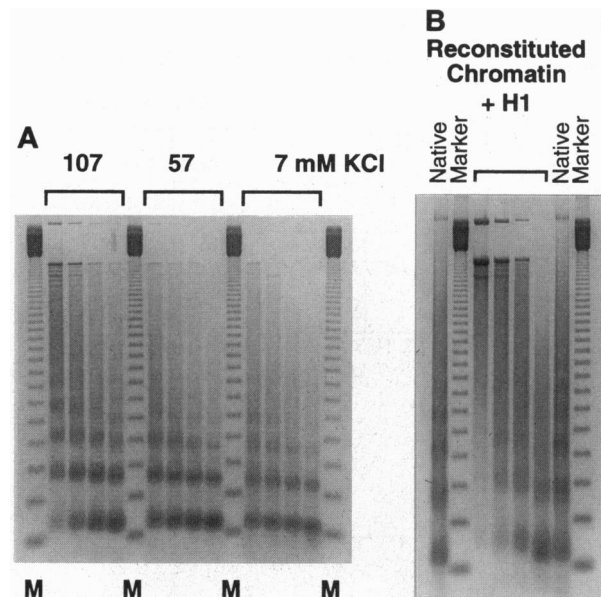


FIG. 2. The nucleosomal repeat length is affected by monovalent salt concentration and histone H1. For each reaction condition, the different lanes represent increasing concentrations (from left to right) of micrococcal nuclease used to digest the chromatin as described in text. (A) Modulation of nucleosomal spacing by monovalent salt concentration in the absence of histone H1. Assembly reactions, which contain 7 mM KCl in the reaction medium, were performed with the indicated concentrations of additional KCl, and the resulting chromatin was then subjected to micrococcal nuclease digestion analysis. (B) Chromatin assembled with histone H1 possesses a repeat length that resembles that of native *Drosophila* chromatin. The reaction was performed in the presence of 1.5 molecules of histone H1 per 200 bp of DNA, and the chromatin was subjected to micrococcal nuclease digestion analysis. The same results were obtained with 1.0–2.5 molecules of H1 per 200 bp of DNA in the assembly reaction (unpublished data). The native chromatin standard was prepared by micrococcal nuclease treatment of nuclei from *Drosophila* embryos. The molecular mass marker (lanes M) is the 123-bp ladder (GIBCO/BRL).

dCAF-1 fraction is the *Drosophila* homologue of human CAF-1. Moreover, with the highly purified *Drosophila* CAF-1 preparation, it is evident that the efficient and rapid assembly of chromatin requires dCAF-1, dCAF-4, and ATP. Yet, because the dCAF-1 has not been purified to homogeneity, it remains possible that there are other chromatin assembly factors in the dCAF-1 fraction.

**dCAF-4 Is a 56-kDa Core-Histone-Binding Protein.** We then examined whether proteins in the dCAF-4 fraction contained core-histone-binding activity by glycerol-gradient sedimentation analysis. Examination of 12 independent sedimentation analyses (with five different dCAF-4 preparations), 1 of which is depicted in Fig. 4A, consistently revealed that a predominant protein in dCAF-4 with an apparent molecular mass of 56 kDa, termed p56, sediments in the upper-middle region of the gradient in the absence of core histones (Fig. 4A Upper, fractions 6 and 7) but comigrates with all of the four core histones to a lower region of the gradient when dCAF-4 was combined with the histones (Fig. 4A Lower, fractions 8 and 9). For comparison, the purified core histones remain at the top of the gradient (fractions 1 to 3) in the absence of dCAF-4 (unpublished data). In addition, at least one minor component of dCAF-4, such as the polypeptide with an apparent molecular mass of 120 kDa, also appeared to comigrate with the core histones in fractions 8 and 9, but only a fraction of this protein shifted with the core histones, which is in contrast to the quantitative shift observed with p56.

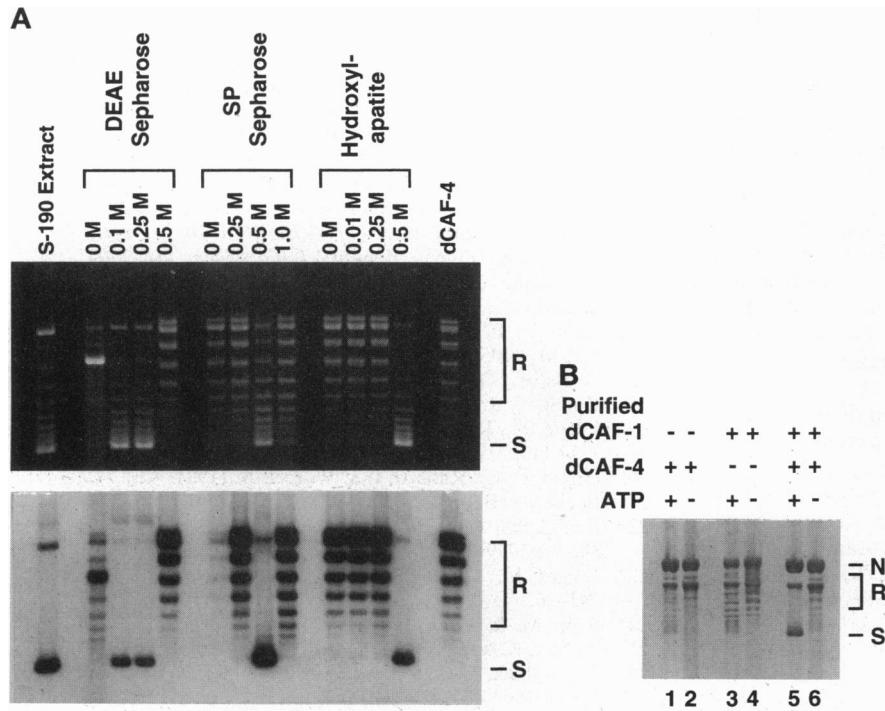


FIG. 3. dCAF-1 appears to be the *Drosophila* homologue of human CAF-1. (A) Identification of CAF-1 activity in fractions derived from the *Drosophila* S-190 extract by using a DNA replication assay with a CAF-1-deficient, human 293 cell extract (12, 13). T antigen- and SV40 origin-dependent DNA replication reactions were performed with the S-190 fractions outlined in Fig. 1A. The deproteinized DNA samples were subjected to 1% agarose gel electrophoresis. (A Upper) Total DNA was visualized by staining with ethidium bromide. (A Lower) Newly replicated DNA was visualized by autoradiography (the replicated DNA was radiolabeled by the inclusion of [ $\alpha$ - $^{32}$ P]dATP in the reaction). (B) Nonreplicative chromatin assembly by dCAF-4 and purified dCAF-1. Assembly reactions were carried out for 4 hr in the presence of the indicated factors, and the resulting chromatin was subjected to DNA supercoiling analysis. The CAF-1 used in these reactions was purified from *Drosophila* embryos to  $\approx$ 30% homogeneity by using the replicative CAF-1 assay.

To determine whether the fast-sedimenting core histone-dCAF-4 complexes obtained by glycerol-gradient sedimentation were functionally active for chromatin assembly, we

carried out a series of reactions with purified dCAF-1, ATP, and each of the glycerol-gradient fractions shown in Fig. 4A Lower. This experiment, which is presented in Fig. 4B, revealed

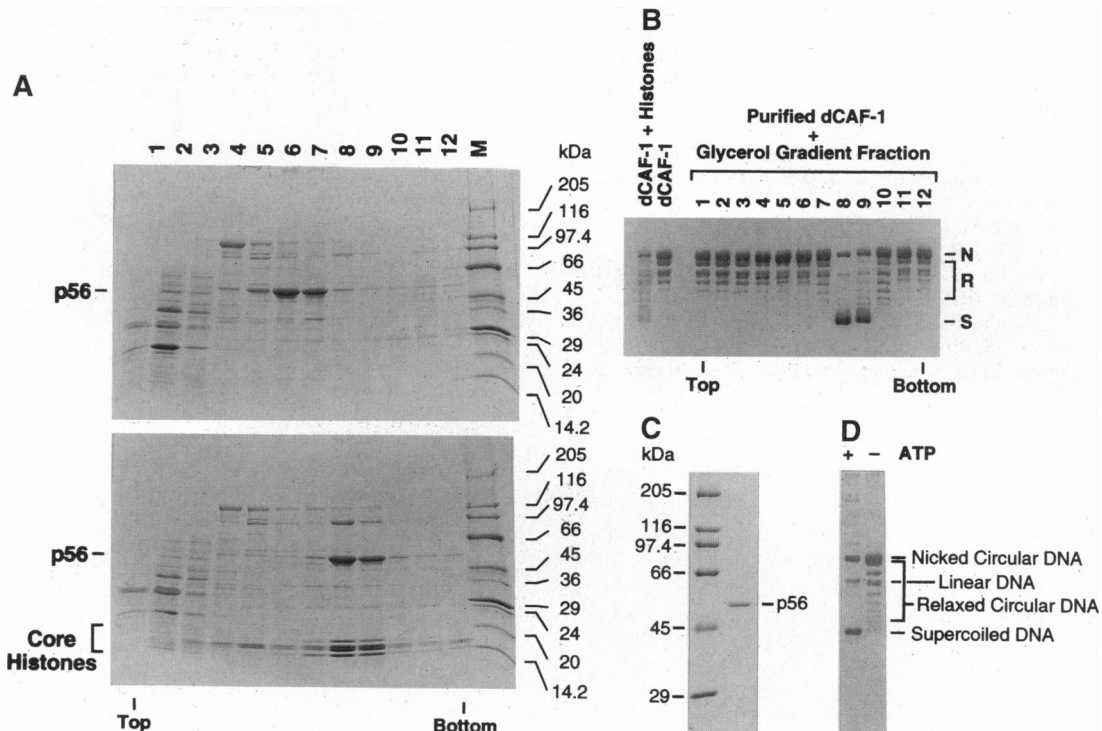


FIG. 4. dCAF-4 is a 56-kDa core-histone-binding protein. (A) A predominant 56-kDa protein in the dCAF-4 fraction comigrates with the four core histones during glycerol gradient sedimentation. The dCAF-4 fraction was sedimented through glycerol gradients in the absence (Upper) or the presence (Lower) of purified core histones. The fractions were then subjected to electrophoresis in 4–25% (wt/vol) polyacrylamide gradient gels containing SDS, and the proteins were visualized by staining with Coomassie brilliant blue R-250. The positions of molecular mass markers, core histones, and p56 are indicated. (B) The fast-sedimenting dCAF-4-core histone complexes are active for chromatin assembly, as determined by DNA supercoiling analysis. Assembly reactions were performed with relaxed circular DNA, ATP, purified dCAF-1, and the indicated glycerol-gradient fractions from the experiment shown in A Lower. In the leftmost lane, core histones were included at a histone/DNA ratio of 0.83:1 (wt/wt). (C) Analysis of purified dCAF-4 by 10% (wt/vol) polyacrylamide/SDS gel electrophoresis. Protein was visualized by staining with Coomassie brilliant blue R-250. (D) Assembly of chromatin by dCAF-1 and purified p56/dCAF-4 as determined by DNA supercoiling analysis. Chromatin assembly reactions were carried out for 4 hr with dCAF-1 ( $\approx$ 30% purity), the preparation of purified p56/dCAF-4 that is shown in C, relaxed circular DNA, and purified *Drosophila* core histones in either the presence or the absence of ATP as indicated.

that the glycerol-gradient fractions containing the fast-sedimenting core histone-p56 complexes were able to substitute for both dCAF-4 and core histones in the assembly reaction.

We therefore purified p56 to >90% homogeneity by ammonium sulfate precipitation and chromatography on butyl-Sepharose (Pharmacia Biotech) (Fig. 4C). The activity of the purified p56 was indistinguishable from that of the dCAF-4 fraction, as determined by the DNA supercoiling assay (Fig. 4D) and the micrococcal nuclease digestion assay (unpublished data). Hence, p56/dCAF-4 is a *Drosophila* core-histone-binding protein, and it may be related to histone-binding factors, such as nucleoplasmin, N1/N2, or NAP-I, which have been characterized in *Xenopus*, yeast, and humans.

**Summary and Perspectives.** In this study, we have been able to assemble chromatin *in vitro* efficiently when dCAF-1, p56/dCAF-4, histones, DNA, and ATP are all present, and the results suggest that the assembly of chromatin by dCAF-1 and dCAF-4 is an ATP-dependent process that yields regularly spaced nucleosomal arrays with high efficiency. It is our hope that these studies will lead to a better understanding of the function of assembly factors in the context of DNA replication and cell growth and in the diverse biological processes that are dependent upon both the integrity and the plasticity of chromatin structure.

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