MOLECULAR AND CELLULAR BIOLOGY, June 1996, p. 3112–3124
0270-7306/96/$04.00+0

Vol. 16, No. 6

Drosophila NAP-1 Is a Core Histone Chaperone That Functions in ATP-Facilitated Assembly of Regularly Spaced Nucleosomal Arrays

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Received 2 February 1996/Returned for modification 11 March 1996/Accepted 20 March 1996

We describe the cloning and analysis of *Drosophila* nucleosome assembly protein 1 (dNAP-1), a core histone-binding protein that functions with other chromatin assembly activities in a *Drosophila* chromatin assembly factor 1-containing fraction (dCAF-1 fraction) in the ATP-facilitated assembly of regularly spaced nucleosomal arrays from purified core histones and DNA. Purified, recombinant dNAP-1 acts cooperatively with a factor(s) in the dCAF-1 fraction in the efficient and DNA replication-independent assembly of chromatin. In the presence of histone H1, the repeat length of the chromatin is similar to that of native chromatin from *Drosophila* embryos. By coimmunoprecipitation analysis, dNAP-1 was found to be associated with histones H2A and H2B in a crude whole-embryo extract, which suggests that dNAP-1 is bound to the histones in vivo. Studies of the localization of dNAP-1 in the *Drosophila* embryo revealed that the factor is present in the nucleus during S phase and is predominantly cytoplasmic during G_2 phase. These data suggest that NAP-1 acts as a core histone shuttle which delivers the histones from the cytoplasm to the chromatin assembly machinery in the nucleus. Thus, NAP-1 appears to be one component of a multifactor chromatin assembly machinery that mediates the ATP-facilitated assembly of regularly spaced nucleosomal arrays.

Chromatin assembly is a process that interfaces DNA replication, gene expression, and progression through the cell cycle, and it is therefore critically involved in many important biological phenomena (for reviews, see references 9, 20, 48, 51, 54, and 58). Nucleosome assembly appears to occur by the initial deposition of histones H3 and H4 with the subsequent incorporation of H2A and H2B in a manner that results in the random distribution of the old preexisting histones and newly synthesized histones between the daughter DNA strands. Analysis of chromatin assembly in vivo has revealed that assembly commences immediately after DNA replication (37, 38), but the kinetics of chromatin maturation (see, for example, references 45 and 59) as well as the observation of chromatin assembly in the apparent absence of DNA replication (upon inhibition and subsequent reinduction of histone synthesis [31]) suggests that assembly need not be directly coupled to DNA replication. In agreement with these data, recent biochemical studies of DNA replication and chromatin assembly have suggested that there may be indirect mechanisms for the coupling of replication and assembly (27). Therefore, it appears that chromatin assembly commences immediately after replication but might not be directly linked to the replication process.

Biochemical analysis of chromatin assembly has led to the identification of core histone-binding proteins, such as nucleoplasmin, N1/N2, and NAP-1, which appear to function as histone transfer vehicles that facilitate the random deposition of histones onto DNA in an ATP-independent manner (for reviews, see references 9, 20, 51, 54, and 58). In addition, in an apparently analogous fashion, polyanions, such as polyglutamic acid (49) or RNA (40), can function as negatively charged, histone transfer molecules. Because the chromatin that is reconstituted with the purified polyanionic species (the histone-binding proteins, polyglutamate, or RNA) consists of randomly distributed nucleosomes and does not exhibit the approximately regular spacing of nucleosomes that is seen with native chromatin, it has been generally thought that at least some of the previously identified core histone-binding proteins are likely to be involved in chromatin assembly but also that the histone-binding proteins are probably not fully responsible for the assembly of chromatin that is observed in vivo.

In studies of DNA replication and chromatin assembly, a factor termed chromatin assembly factor 1 (CAF-1) was found to be required for the assembly of newly replicated DNA into chromatin during T antigen-mediated simian virus 40 DNA replication in vitro (47, 50). In those experiments, CAF-1 activity was identified by complementation of a CAF-1-deficient, cytosolic replication extract derived from human 293 cells. Hence, CAF-1-mediated chromatin assembly occurs in conjunction with other factors, which have not yet been identified, which are present in the 293 cell extract. By using the simian virus 40 DNA replication assembly assay, CAF-1 was purified and cloned from human cells (28, 47). The largest subunit (p150) of human CAF-1 appears to bind to newly synthesized H3 and H4 (28, 46), but, unlike the core histone-binding proteins such as nucleoplasmin or NAP-1, purified CAF-1 alone does not possess significant nucleosome deposition activity. In addition, analysis of the properties of human and *Drosophila* CAF-1 has led to the finding that chromatin assembly by CAF-1 can occur postreplicatively (27). However, in those studies, it was found that the postreplicative assembly of chromatin by CAF-1 occurs preferentially with newly-replicated DNA relative to unreplicated DNA. Nevertheless, these biochemical studies with CAF-1 are consistent with the results of in vivo analyses of chromatin assembly, which indicate that assembly occurs with newly replicated DNA but does not necessarily require concomitant DNA replication.

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Crude extracts derived from *Drosophila*, *Xenopus*, or mammalian cells are able to mediate ATP-facilitated and DNA replication-independent assembly of chromatin that exhibits approximately regular spacing of nucleosomes, as is seen with native chromatin (see, for instance, references 1, 2, 19, and 26). Fractionation of the *Xenopus* extracts led to the identification of nucleoplasmin and N1/N2, which were able to mediate the ATP-independent, random deposition of nucleosomes (see, for example, references 32, 35, 42, 43, 53, 60). In addition, it was found that phosphorylated HMG17 affects the nucleosome spacing of chromatin that is reconstituted with H2A-H2B dimer and N1/N2-H3-H4 complexes (12, 52). However, in the earlier experiments, chromatin reconstitution with the purified/fractionated components did not resemble the ATP-facilitated assembly of extended nucleosomal arrays by the full complement of factors in crude assembly extracts with regard to (i) the requirement for ATP for efficient chromatin assembly; (ii) the regularity of the nucleosomal spacing; and (iii) the nucleosome repeat length (the repeat length of chromatin reconstituted with purified components typically ranged from 145 to 165 bp, depending on the conditions). Hence, further studies were necessary to identify the factors that mediate the ATP-facilitated assembly of nucleosomal arrays.

We had previously fractionated the chromatin assembly activities in a crude *Drosophila* embryo extract and obtained two partially purified fractions that were termed dCAF-1 and dCAF-4 (for *Drosophila* chromatin assembly factors/fractions 1 and 4), which were able to mediate the assembly of regularly spaced nucleosomal arrays when combined with DNA, purified core histones, and ATP (6). The dCAF-1-containing fraction was thus so designated because this fraction was observed to possess the *Drosophila* version of chromatin assembly factor 1 (CAF-1) (6). (This nomenclature is used with some trepidation, however, because it has yet to be established whether the *Drosophila* version of CAF-1 is an active component of that fraction.) Characterization of the dCAF-4 fraction led to the identification of a 56-kDa core histone-binding protein, designated p56, which is the primary focus of this study. The nucleosome repeat length of chromatin assembled by these fractions in the presence of histone H1 was identical to that of native chromatin. Thus, the data collectively suggest that the ATP-facilitated assembly of chromatin by the fractionated factors resembles, at least in part, the natural mechanism of chromatin assembly.

In this report, we describe the cloning of the p56 component of dCAF-4 and the finding that it is the *Drosophila* homolog of the core histone-binding protein, NAP-1 (21, 22). Biochemical characterization of *Drosophila* NAP-1 (dNAP-1) as well as analysis of the subcellular localization of dNAP-1 in the embryo at different periods of the cell cycle leads to a model for chromatin assembly wherein NAP-1 functions to deliver core histones to the chromatin assembly machinery.

MATERIALS AND METHODS

Isolation of cDNA encoding p56 (dNAP-1). The p56 core histone-binding protein in the dCAF-4 fraction was purified to greater than 90% homogeneity from the *Drosophila* S-190 chromatin assembly extract (5, 26) by sequential chromatography on DEAE-Sepharose (Pharmacia), SP-Sepharose (Pharmacia), Q-Sepharose (Pharmacia), and butyl-Sepharose (Pharmacia) resins as described by Bulger et al. (6). The p56 sample was subjected to electrophoresis on a 12% polyacrylamide-sodium dodecyl sulfate (SDS) gel, which was then stained with Coomassie blue G. The p56 band was excised, and the protein was digested with a lysylendopeptidase (*Achromobacter* protease I; Wako Chemicals). The resulting peptides were purified by high-performance liquid chromatography with a C-18 column (Vydac) and sequenced by automated Edman degradation (Applied Biosystems model 470, 473, or 477). A fragment of the cDNA was obtained by using reverse transcription-PCR with oligonucleotides corresponding to the coding sequences of the peptides, and six independent cDNAs were isolated by screening a *Drosophila* embryo cDNA library (embryos were collected from 0 to 4 h after egg deposition) in λZAPII with the radiolabelled, PCR-amplified fragment. The entire cDNA sequences from both DNA strands of two independent cDNAs were sequenced, and it was evident that p56 was the *Drosophila* homolog of NAP-1. One of these cDNAs was PCR amplified and subcloned into the *Nco*I and *Xho*I sites of pET15b (Novagen) to give the bacterial expression plasmid pETdNAP-1, and the coding region was then completely resequenced to confirm the absence of mutagenesis occurring during the PCR process.

Purification of recombinant dNAP-1. A freshly transformed colony of pETdNAP-1 in *Escherichia coli* BL21(DE3) was grown at 37°C in a volume of 2 liters to an A_{600} of ~0.6, and synthesis of dNAP-1 was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.4 mM. The culture was incubated for an additional 3 h at 30°C. Unless stated otherwise, all subsequent operations were performed at 4°C. The bacteria were pelleted by centrifugation (Sorvall GSA rotor; 5,000 rpm; 5 min) and resuspended in 40 ml of buffer R (10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; K^+; pH 7.6] containing 10 mM KCl, 1.5 mM $MgCl_2$, 10% glycerol, 10 mM β-glycerophosphate, 1 mM dithiothreitol, and 0.2 mM phenylmethylsulfonyl fluoride). The bacteria were lysed by sonication, and the insoluble material was removed by centrifugation (Sorvall SS-34 rotor; 10,000 rpm; 10 min). Then, 4 M ammonium sulfate (pH 7.0) was added to the supernatant to a final concentration of 2.25 M. The mixture was incubated for 20 min and then subjected to centrifugation (Sorvall SS-34 rotor; 10,000 rpm; 10 min). The supernatant was applied to a phenyl-Sepharose CL-4B (Pharmacia) column (column volume, 30 ml; column dimensions [diameter by length], 1.6 by 15 cm; flow rate, 1 ml/min; fraction size, 5 ml). The column was washed with 3 column volumes of buffer R containing 2.25 M ammonium sulfate, and protein was eluted with a linear gradient (10 column volumes) of from 2.25 to 0 M ammonium sulfate. Fractions were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE), and the peak dNAP-1-containing fractions were pooled and dialyzed against buffer R containing 0.2 M NaCl. The resulting sample was applied onto tandemly linked P11 (phosphocellulose [Whatman]; column volume, 30 ml; column dimensions [diameter by length] = 1.6 by 15 cm; flow rate, 0.2 ml/min) and Mono Q columns (HR 5/5 [Pharmacia]) (note that the P11 column was first and the Mono Q was second). After application of the protein sample, the P11 column was removed, and the Mono Q resin was washed with 4 column volumes of buffer R containing 0.2 M NaCl. dNAP-1 was eluted with a linear gradient (10 column volumes) of from 0.2 to 1 M NaCl in buffer R. dNAP eluted from the Mono Q resin at roughly 250 mM NaCl. The protein content of the fractions was analyzed by SDS-12% PAGE. Nuclease activity in the fractions was detected by incubation of plasmid DNA with the samples at 37°C for 1 h in the presence of 10 mM $MgCl_2$ and then by analysis of the DNA by 1% agarose gel electrophoresis. Bacterial nucleic acid contamination was monitored by 1% agarose gel electrophoresis and staining with ethidium bromide. The peak dNAP-1-containing fractions, which were not contaminated with nucleases or bacterial nucleic acids, were dialyzed against buffer R (with no additional salt), frozen in liquid nitrogen, and stored at −80°C.

Assembly and analysis of chromatin. Chromatin assembly reactions were performed under the conditions (at 57 mM KCl) described by Bulger et al. (6) by using circular plasmid DNA (470 ng, previously relaxed by incubation with purified, recombinant *Drosophila* topoisomerase I), dCAF-1 fraction (19 μg of total protein from the 0.25 to 0.5 M potassium phosphate fraction of the hydroxylapatite column [6]), purified recombinant dNAP-1 (3.2 μg), purified *Drosophila* core histones (410 ng), 3 mM ATP, and an ATP regeneration system (30 mM phosphocreatine and 1 μg of creatine phosphokinase per ml). The nucleosome repeat length of the assembled chromatin has been observed to vary with the ionic strength of the reaction medium (5, 55), and, hence, as a matter of reference, these reactions were performed under the 57 mM KCl conditions described by Bulger et al. (5), whereby a 162-bp repeat length was previously reported in the absence of H1. Under these conditions, the NAP-1-to-core histone ratio is approximately 2.5:1 (or alternatively, the NAP-1-to-octamer ratio is 20:1), and there is hence an excess of NAP-1 relative to the core histones. We have found that the quality of the chromatin assembled at a NAP-1-to-histone ratio of 1.25:1 is similar to that assembled at a ratio of 2.5:1, whereas a distinctly lower amount of chromatin assembly occurs at a NAP-1-to-histone ratio of 0.63:1 (data not shown). When indicated, purified *Drosophila* histone H1 (8) was included in the reaction mixtures. DNA supercoiling and micrococcal nuclease digestion assays were carried out as described previously (5, 6, 26).

Because of the apparently low levels of assembly that were observed in the absence of dNAP-1, the dCAF-1 fraction, or ATP, we have refrained from a description of the chromatin assembly reaction as being strictly dependent on dNAP-1, dCAF-1, or ATP. It is possible that chromatin assembly is not an all-or-nothing process. Alternatively, there may be a low level of cross-contamination of the factors, such as a trace of dNAP-1 present in the dCAF-1 fraction. However, these data indicate that the efficient assembly of regularly spaced (as opposed to randomly distributed or closely packed) nucleosomal arrays does require dNAP-1, the dCAF-1 fraction, and ATP.

It is also pertinent to note that in studies of NAP-1 by Ishimi and colleagues (16, 21–25), the efficiency of histone deposition by NAP-1 alone appeared to be greater than that which we have observed in the present work and in previous work (6). Consequently, we compared the reaction conditions reported in the most recent description of nucleosome reconstitution by NAP-1 from Fujii-

Nakata and coworkers (16) with those employed in the present study and identified some of the following differences: (i) monovalent salt concentration of 57 mM KCl (the present study) versus 150 mM NaCl (16); (ii) reaction temperature of 27°C (the present study) versus 37°C (16); (iii) use of dNAP-1 (the present study, except for Fig. 3B) versus yeast NAP-1 (16); and (iv) use of a core histone-to-DNA mass ratio of 0.87 (the present study; this mass ratio in chromatin corresponds to roughly 1 histone octamer per 184 bp of DNA) versus 1.5 to 3.0 (16 [this mass ratio in chromatin corresponds to about 1 histone octamer per 53 to 106 bp of DNA]). It is likely that some, if not all, of these differences contribute to the greater extent of nucleosome reconstitution by NAP-1 (in the absence of the dCAF-1 fraction), as observed in previous work (16, 21–25).

Coimmunoprecipitation of dNAP-1 and core histones. A crude *Drosophila* embryo extract was prepared by homogenization of embryos (collected from 0 to 2 h after egg deposition) with a 10-ml Wheaton Dounce homogenizer in buffer R containing 100 mM KCl, which was then followed by sonication. For each immunoprecipitation, crude extract prepared from approximately 300 mg of embryos was used. First, immunoaffinity-purified rabbit anti-dNAP-1 polyclonal antibody was incubated with 70 μl of a 50% (vol/vol) slurry of protein A-Sepharose (Pharmacia) at 4°C for 2 h in buffer R containing 100 mM KCl. The embryo extract was then added, and the mixture was incubated at 4°C for 4 h. The protein A-Sepharose beads were then washed twice with dilution buffer (10 mM Tris-HCl [pH 7.9], 0.14 M NaCl, 0.1% Triton X-100, 0.1% bovine serum albumin), once with TSA (10 mM Tris-HCl [pH 7.9], 0.14 M NaCl), and once with 50 mM Tris-HCl (pH 7.9). The beads were then suspended in SDS sample buffer (40 μl) and boiled for 5 min. A portion of the supernatant was then subjected to SDS-PAGE and Western blot (immunoblot) analysis. For the Western blot analysis of dNAP-1, a mouse anti-NAP-1 monoclonal antibody (clone 4A8 [22]) was used as the primary antibody, and an alkaline phosphatase-conjugated antimouse antibody was used as the secondary antibody. For the Western blot analysis of core histones, an immunoaffinity-purified, biotin-conjugated, rabbit polyclonal antibody prepared against *Drosophila* core histones was used as the primary antibody, and the ABC reagents (Vector) were used for detection of the core histones.

Immunostaining of *Drosophila* embryos. *Drosophila* embryos (collected from 0 to 12 h after egg deposition) were dechorionized and suspended in buffer B (10 mM potassium phosphate [pH 6.8], 15 mM NaCl, 45 mM KCl, 2 mM $MgCl_2$). The embryos were then permeabilized by methodology similar to that described previously (36). All operations were at room temperature, except when stated otherwise. Specifically, the embryos were incubated with shaking for 12 min in a suspension of 3.6 ml of buffer B, 0.4 ml of formaldehyde (37%), and 4 ml of heptane. The lower aqueous phase was removed, and then 8 ml of methanol was added. The embryos were shaken vigorously for 1 min, after which the devitillinized embryos settled to the bottom of the vessel. The upper phase was removed along with embryos at the organic-aqueous interface. The devitillinized embryos were rehydrated with 1:1 (vol/vol) methanol-PBT (PBT is 1× phosphate-buffered saline [PBS] containing 0.1% Tween 80) for 10 min, which was then followed by 30 min of incubation in PBT. The embryos were then incubated for four times of 30 min each in 10% bovine serum albumin in 1× PBS. Then, the primary antibody (anti-NAP-1 monoclonal antibody 4A8 [22]) in PNBT (1× PBS, 0.5 M NaCl, 1% bovine serum albumin, 0.1% Tween 80) was incubated with the embryos overnight at 4°C. The embryos were washed at room temperature for six times of 15 min each with PNBT. The second antibody (sheep anti-mouse immunoglobulin G conjugated with fluorescein), along with 20 μg of propidium iodide per ml (for DNA staining), was incubated with the embryos overnight at 4°C. The embryos were washed at room temperature for six times of 15 min each with PNBT and then analyzed by confocal microscopy. Confocal images were collected, with the generous guidance of Theo Palmer, by using a Bio-Rad MRC 1000 confocal scanning laser head attached to a Zeiss Axiovert 135 M microscope. The data shown were representative of the consistently and reproducibly observed characteristics of many embryos.

Nucleotide sequence accession number. The dNAP-1 sequence has been deposited in GenBank under accession number U39553.

RESULTS AND DISCUSSION

Isolation of cDNA encoding chromatin assembly factor p56 (dNAP-1). To investigate the function of the p56 component of the dCAF-4 fraction in the ATP-facilitated assembly of nucleosomal arrays, we sought to isolate the cDNA encoding the factor. The protein was purified to near homogeneity from *Drosophila* embryos and then subjected to amino acid sequencing analysis. On the basis of the partial amino acid sequence of p56, we obtained six independent cDNAs encoding p56, and upon DNA sequencing of two of the cDNAs, we found that p56 was the *Drosophila* homolog of a core histone-binding protein that is termed nucleosome assembly protein 1 (NAP-1 [21, 22]) (Fig. 1).

NAP-1 (which has also been termed AP-I) was originally identified as a core histone-binding protein in mammalian cells that facilitated random, ATP-independent nucleosome deposition (21, 25), as monitored by the supercoiling of DNA that occurs upon the formation of nucleosomes (18). However, micrococcal nuclease digestion analysis of the nucleoprotein structures that were generated by core histones and NAP-1 did not yield the characteristic extended ladder that is seen with native chromatin (41). Subsequent studies of NAP-1 led to the generation of monoclonal antibodies against the protein and the finding that NAP-1 appears to be in the nuclei of HeLa cells (24), the observation that NAP-1 has a higher affinity for H2A and H2B than for H3 and H4 by in vitro binding studies (23), the isolation of a cDNA encoding NAP-1 from *Saccharomyces cerevisiae* (22), biochemical characterization of mutant versions of NAP-1 (16), and the finding that NAP-1 can facilitate binding of transcription factors to mononucleosomes in vitro (57). In addition, the *NAP1* gene was found to be unessential for viability of *S. cerevisiae* (29), which may be due to the presence of other functionally redundant NAP-1-related proteins, such as SET, which has been found in both humans (56) and *Drosophila melanogaster* (29). The ability of purified NAP-1 to deposit nucleosomes randomly had suggested a role for NAP-1 in the assembly of nucleosomes, which had led to the view that NAP-1 functions by itself to form nucleosomes randomly as a nonspecific histone transfer vehicle. We have found, in contrast, that NAP-1 is a single component of a multifactor chromatin assembly machinery that mediates the ATP-facilitated assembly of regularly spaced nucleosomal arrays.

Examination of the primary amino acid sequence of dNAP-1 (Fig. 1A) revealed that it contains a highly acidic segment near the C terminus, which resembles the C-terminal acidic region of nucleoplasmin (7, 10), that may be involved in the binding of histones. In addition, there is a stretch of amino acid residues (positions 240 to 258 in Fig. 1A) that has characteristics similar to those of nuclear localization signals (4, 11) as well as a segment (positions 57 to 65 in Fig. 1A) that resembles a nuclear export signal (17). Comparison of the primary amino acid sequences of NAP-1 from different organisms revealed 47% identity between dNAP-1 versus human NAP-1 and 31% identity between dNAP-1 versus yeast NAP-1 (Fig. 1B). It is also apparent that the basic residues of the putative nuclear import signal, the leucine/isoleucines of the putative nuclear export signal, and the acidic amino acid stretch are each well-conserved features of NAP-1. Furthermore, there are three highly conserved motifs, KGIPXFWLT (positions 207 to 215 of Fig. 1B), DSFFNFFXPP (positions 340 to 349 of Fig. 1B), and DFEIGXXLRXRIIPKAVXYYTG (positions 373 to 394 of Fig. 1B), of which the first two had been identified previously (44), that may also be important for the function of NAP-1.

Northern (RNA) blot analysis with poly(A)$^+$ RNA from *Drosophila* embryos (0 to 12 h after fertilization) revealed a single band of approximately 1.5 kb (data not shown), which corresponds well with the length of the cDNAs [~1.3 kb, not including the poly(A) tail]. Southern blot analysis indicated that dNAP-1 is encoded by a single gene (data not shown). By in situ hybridization to polytene chromosomes, the dNAP-1 locus was mapped to region 60A, bands 5 and 6, on the right arm of the second chromosome (35a).

Chromatin assembly with recombinant dNAP-1. To characterize further the biochemical properties of dNAP-1, it was important to establish whether purified recombinant dNAP-1 possessed chromatin assembly activity that is similar to that of the dCAF-4 fraction from which dNAP-1 was originally derived. Therefore, we synthesized dNAP-1 in *Escherichia coli*

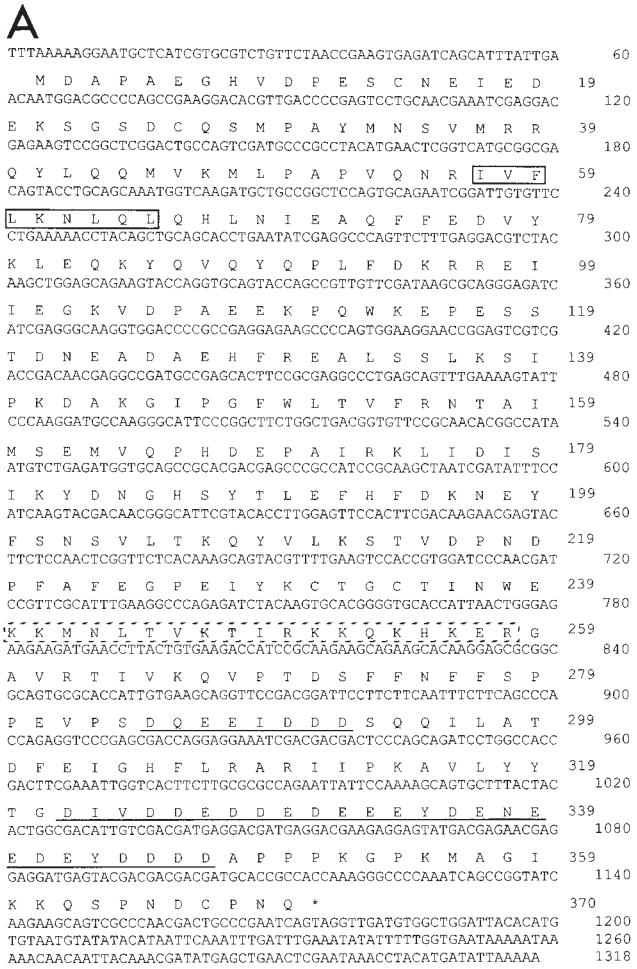


FIG. 1. The 56-kDa core histone-binding protein in the dCAF-4 fraction is dNAP-1. (A) Nucleotide sequence of a cDNA encoding p56/dNAP-1. The predicted amino acid sequence is given in the single-letter amino acid code. A region that may contain a nuclear localization signal (4, 11) is encompassed by a box with dashed lines, whereas a sequence that resembles a nuclear export signal (17) is denoted by a box with solid lines. Stretches of acidic amino acid residues are underlined. (B) Alignment of the amino acid sequences of NAP-1 from humans (H), mice (M), sea urchins (S), *D. melanogaster* (D), *Arabidopsis thaliana* (A), and *S. cerevisiae* (Y). The stars indicate identical or conserved amino acid residues.

and purified the recombinant protein to greater than 95% homogeneity (Fig. 2A). The recombinant dNAP-1 was then tested for its ability to mediate chromatin assembly in conjunction with the dCAF-1 fraction in reaction mixtures that also contained DNA, core histones, and ATP (Fig. 2B and C). DNA supercoiling and micrococcal nuclease digestion analyses revealed that the efficient assembly of extended nucleosomal arrays can be mediated by recombinant dNAP-1, the dCAF-1 fraction, and ATP. Thus, the nucleosome assembly activity of purified recombinant dNAP-1 is indistinguishable from that which has been described for the partially purified dCAF-4 fraction (5).

We also tested whether purified recombinant dNAP-1 functions cooperatively with a factor(s) in the dCAF-1 fraction. To this end, we carried out a set of reactions containing either the normal amount (i.e., the quantity of each factor used in a standard assembly reaction, as described in Materials and Methods, which is designated 1×) of dNAP-1 and/or the dCAF-1 fraction or twice the normal amount (i.e., 2×) of dNAP-1 or the dCAF-1 fraction. As shown in Fig. 2D, the inclusion of 2× the concentration of either dNAP-1 alone or the dCAF-1 fraction alone has little effect on the efficiency of chromatin assembly relative to that seen with the normal concentration of either factor (Fig. 2D; compare lane 2 with lane 5 and lane 3 with lane 6), while in contrast, the combination of 1× dNAP-1 with 1× the dCAF-1 fraction results in a significant increase in chromatin assembly (Fig. 2D, lane 4). Therefore, we conclude that dNAP-1 and a factor(s) in the dCAF-1 fraction function cooperatively in the assembly of chromatin.

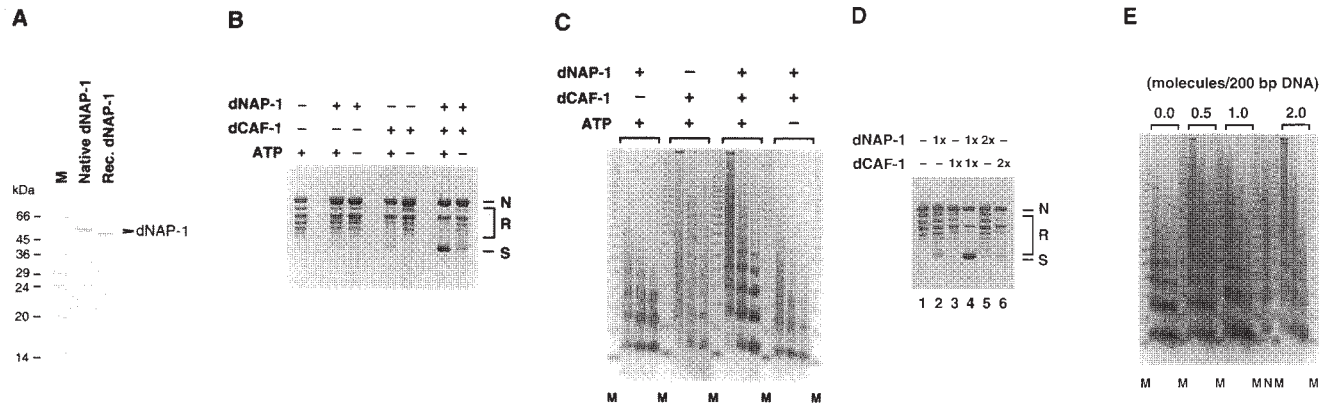


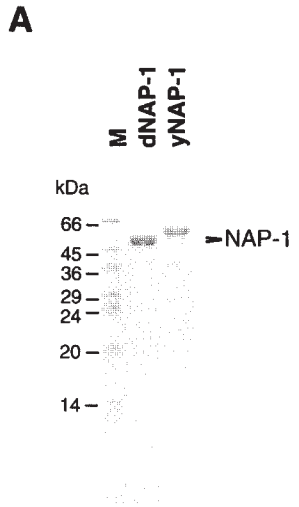
FIG. 2. Assembly of chromatin with purified, recombinant dNAP-1 and the native dCAF-1 fraction. (A) Purification of *E. coli*-synthesized dNAP-1. Purified, native dNAP-1 (p56 of the dCAF-4 fraction) and recombinant dNAP-1 were analyzed by SDS-12% PAGE and staining with Coomassie blue R-250. Native NAP-1 from *Drosophila* embryos migrates slightly more slowly than the bacterially synthesized protein. The basis for this difference in electrophoretic migration rates is not known but may be due to some form of posttranslational modification of the protein in the embryo. (B) DNA supercoiling analysis. Chromatin assembly reactions were carried out with relaxed, circular DNA (3.25-kbp plasmid) and purified core histones in the presence or absence of recombinant dNAP-1, the dCAF-1 fraction, or ATP as indicated. After 4 h, 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. Chromatin assembly reactions were carried out in either the presence or the absence of the indicated factors. The samples were partially digested with micrococcal nuclease for various reaction times and then deproteinized. For each reaction condition, the different lanes represent increasing concentrations, from left to right, of micrococcal nuclease used to digest the chromatin. The resulting DNA fragments were subjected to agarose gel electrophoresis and visualized by staining with ethidium bromide. The molecular mass markers (M) are the 123-bp ladder (Gibco-BRL). (D) Examination of synergy between dNAP-1 and the dCAF-1 fraction in the assembly of chromatin, as measured by the DNA supercoiling assay. Chromatin assembly reactions were performed and analyzed as described for panel B. When indicated, either 1× (the amount of factor included in a standard reaction mixture, as described in Materials and Methods) or 2× (twice the amount of factor used in a standard reaction mixture) dNAP-1 or the dCAF-1 fraction was included in the reaction mixtures. (E) Incorporation of histone H1 into chromatin. The indicated amounts of histone H1 (in molecules per 200 bp of DNA) were added prior to chromatin assembly. The native chromatin reference (N) was prepared by treatment of *Drosophila* embryo nuclei with micrococcal nuclease. The molecular mass markers (M) are the 123-bp ladder (Gibco-BRL).

We then examined the incorporation of histone H1 into chromatin (Fig. 2E). When histone H1 was included in the reaction medium prior to the assembly of chromatin, the estimated repeat lengths of the nucleosomal arrays were as follows: 161 bp in the absence of H1, 167 bp with 0.5 molecules of H1 per 200 bp of DNA, 188 bp with 1.0 molecule of H1 per 200 bp of DNA, and 193 bp with 2.0 molecules of H1 per 200 bp. Thus, the regularity of nucleosomal distribution and the repeat length of the H1-containing chromatin (~190 bp) are similar to those of bulk chromatin from *Drosophila* embryos.

In addition, we investigated whether the distantly related NAP-1 from yeast (yNAP-1) would be able to function with dCAF-1 fraction in the assembly of nucleosomes (Fig. 3). yNAP-1 was synthesized in *E. coli* and purified to greater than 95% homogeneity by using methodology similar to that for the purification of dNAP-1. Chromatin assembly reactions were then performed with equimolar amounts of either dNAP-1 or yNAP-1. In the presence of the dCAF-1 fraction, both yNAP-1 and dNAP-1 were able to mediate the efficient assembly of extended nucleosomal arrays. These results suggest that the basic function of NAP-1 in chromatin assembly is conserved between yNAP-1 and dNAP-1. In addition, it should be noted that reactions performed with either yNAP-1 or dNAP-1 alone yielded products that exhibited a significantly shorter repeat length than the nucleosome arrays that were assembled in the presence of both NAP-1 and the dCAF-1 fraction (Fig. 3B; see also Fig. 2B). (This difference in the observed nucleosome repeat lengths has been consistently observed when the reactions were performed in the presence of different salt concentrations as well as upon micrococcal nuclease digestion of the chromatin to various extents [both of these factors could affect the apparent nucleosome repeat length; see, for example, references 3 and 5].) Therefore, it appears that NAP-1 and the dCAF-1 fraction not only function cooperatively for chromatin assembly but also yield chromatin that is different from the reaction product that is generated with NAP-1 alone.

Dynamics of the chromatin assembly reaction. We then examined the reaction rate of chromatin assembly with recombinant dNAP-1. By the DNA supercoiling assay, the reaction appears to be nearly complete by 30 min (data not shown), which appears to be lower than the rate of supercoiling observed with the partially purified dCAF-4 fraction (5). Under identical conditions with the micrococcal nuclease digestion assay, a moderate digestion ladder (at least 5 bands) could be observed in 2 min of reaction time (which, in the assay, is followed by 5 min of digestion time with micrococcal nuclease), while a long, extended ladder (at least 10 distinct bands) was seen after 30 min of reaction time (Fig. 4A). Although the rate of assembly, as measured by micrococcal nuclease digestion analysis, may appear to be more rapid than that monitored by the DNA supercoiling assays, it should be noted in Fig. 4A that the chromatin that was generated in short reaction times was significantly more sensitive to micrococcal nuclease digestion than the more fully assembled chromatin, which indicates that a low overall degree of assembly had occurred in the shorter reaction times. Hence, the data obtained from the two assays appear to be self-consistent. In terms of a reaction mechanism, we interpret these results, particularly the rapid formation of nucleosomal arrays that are observed in the micrococcal nuclease digestion assay, to indicate that the chromatin assembly process commences immediately and does not appear to exhibit any lag period.

We also investigated the specificity of the requirement for ATP to facilitate the assembly reaction. To this end, we carried out assembly reactions with equimolar concentrations of ATP, dATP, GTP, CTP, UTP, and the nonhydrolyzable ATP analog adenylyl imidodiphosphate (AMP-PNP). Because of an apparent ATPase or phosphatase activity in the partially purified



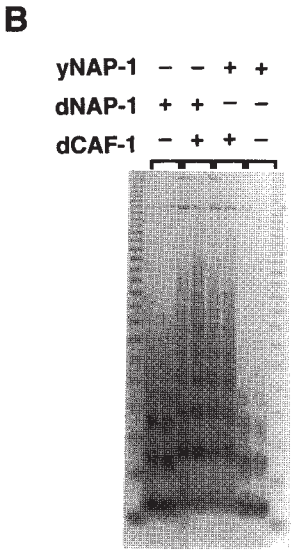


FIG. 3. yNAP-1 can substitute for dNAP-1 in the assembly reaction. (A) Analysis of *E. coli*-synthesized yNAP-1 and dNAP-1 by SDS-12% PAGE and staining with Coomassie blue; (B) micrococcal nuclease digestion analysis with yNAP-1 versus dNAP-1.

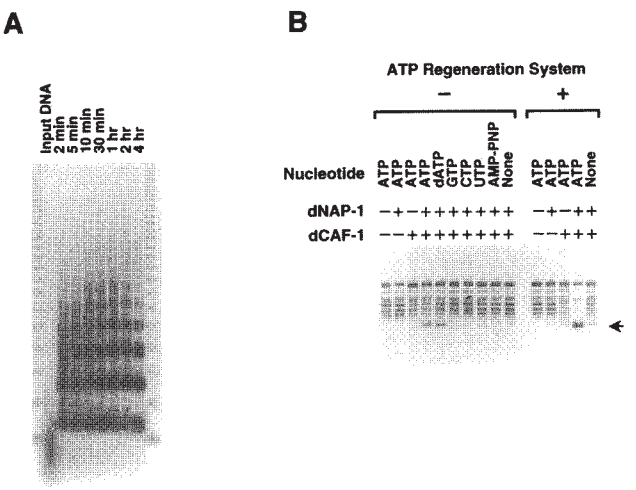


FIG. 4. Dynamics of chromatin assembly by dNAP-1 and the dCAF-1 fraction. (A) Time course of assembly by dNAP-1 and the dCAF-1 fraction, as measured by micrococcal nuclease digestion assaying. Samples were sequentially removed from a single reaction at the indicated times, digested with micrococcal nuclease for 5 min, and deproteinized. The resulting DNA was subjected to 1.5% agarose gel electrophoresis and visualized by staining with ethidium bromide. (B) Nucleotide requirement for chromatin assembly. Chromatin assembly reactions were performed with the indicated nucleotides or nucleotide analogs (3 mM concentration) in the absence (left panel) or the presence (right panel) of an ATP-regenerating system (30 mM phosphocreatine and 1 μg of creatine phosphokinase per ml). The efficiency of chromatin assembly was monitored by the DNA supercoiling assay.

dCAF-1 fraction, the chromatin assembly reactions are typically carried out in the presence of an ATP-regenerating system, which consists of phosphocreatine and creatine phosphokinase (Fig. 4B, right panel). In the absence of the ATP-regenerating system, chromatin assembly is less efficient (Fig. 4B, left panel). Nevertheless, it can be seen in this experiment that chromatin assembly with either ATP or dATP occurs with comparable efficiency, which is significantly greater than the amount of chromatin assembly observed with GTP, CTP, UTP, and AMP-PNP. In addition, the ability of ATP, but not AMP-PNP, to facilitate the reaction suggests that hydrolysis of the β-γ phosphate anhydride bond of ATP is involved during chromatin assembly.

Binding of dNAP-1 to core histones. We then sought to examine the binding of dNAP-1 to core histones. We first carried out glycerol gradient sedimentation analysis of recombinant dNAP-1 in the presence or the absence of purified core histones (Fig. 5). In the absence of histones, dNAP-1 alone appears to be multimeric. The apparent molecular mass of dNAP-1 is approximately 120 kDa as measured by glycerol gradient sedimentation and roughly 600 kDa as estimated by gel filtration (data not shown). The basis of this sharp difference in the apparent molecular mass values of dNAP-1 might be due to some of the following reasons: (i) differences in the buffer media in the two experiments, such as the glycerol concentration; (ii) an extended, nonspherical protein structure; and/or (iii) aberrantly fast migration of dNAP-1 during gel filtration chromatography because of charge repulsion between the negatively charged regions of dNAP-1 and the gel filtration resin. (It might be pertinent to note that a large difference in the apparent molecular masses of human and dCAF-1, as estimated by glycerol gradient sedimentation [~120 kDa] and gel filtration [~700 kDa], has also been observed [27, 46].) In the presence of core histones, dNAP-1 cosedimented with all four core histones. These results indicate that dNAP-1 can associate, either directly or indirectly, with each of the four core histones, and these data are consistent with the findings of earlier studies of the binding of core histones to mammalian NAP-1 (21, 23) or to the dNAP-1-containing chromatin assembly factor 4 (dCAF-4) fraction (5).

Then, we examined whether core histones are associated with dNAP-1 in a crude, whole-embryo extract by immunoprecipitation of dNAP-1 from the extract and then by Western blot analysis with antibodies against histones H2A, H2B, and H3. As shown in Fig. 6, histones H2A and H2B, but not (or to a much lesser extent) H3, coimmunoprecipitate with dNAP-1 from the whole-embryo extract with antibodies against dNAP-1, but not with a control preimmune serum. (It is important to note that the antibodies used in the Western blot shown in the right panel of Fig. 6 recognize H2A, H2B, and H3 but not H4.) As a control, when purified core histones were combined with dNAP-1 in vitro and then subjected to immu-

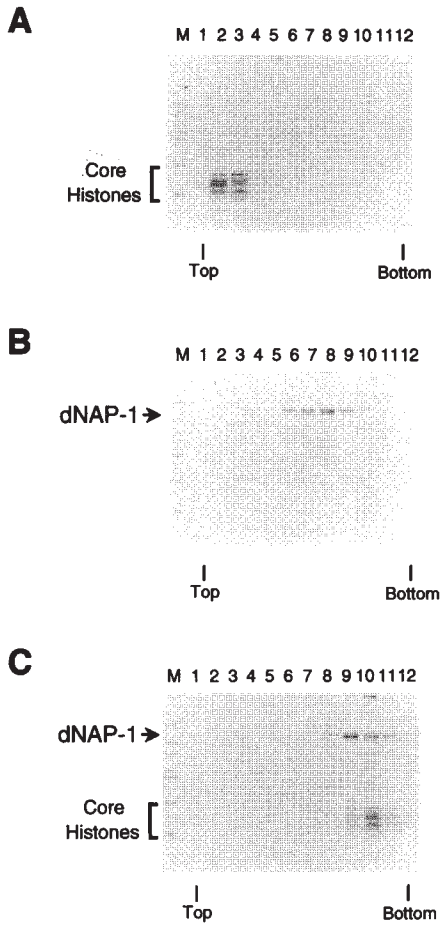


FIG. 5. Recombinant dNAP-1 binds to core histones in vitro. dNAP-1 and core histones were subjected to 15 to 50% glycerol gradient sedimentation in a SW55 (Beckman) rotor at 50,000 rpm for 16 h at 4°C, and the protein fractions were analyzed by SDS-15% PAGE. (A) Core histones only (26 μg); (B) dNAP-1 only (40 μg); (C) core histones (26 μg) and dNAP-1 (40 μg).

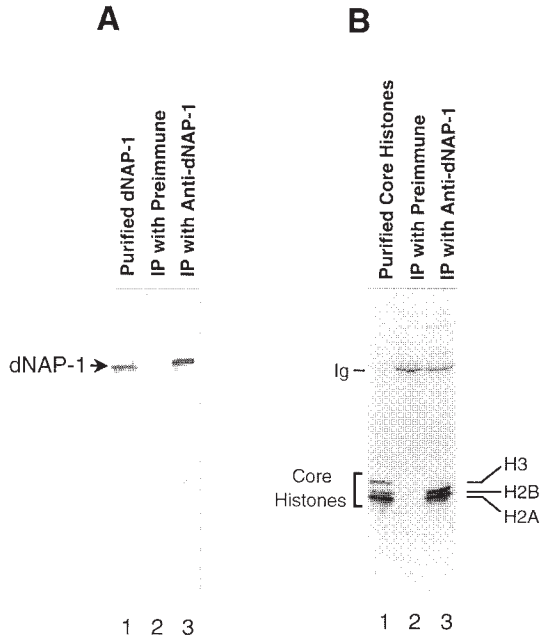


FIG. 6. dNAP-1 binds to H2A and H2B in a crude, whole-embryo extract. (A) Immunoprecipitation (IP) of dNAP-1 from a *Drosophila* whole-embryo extract. A crude extract derived from ~300 mg of *Drosophila* embryos was subjected to immunoprecipitation with either preimmune serum or anti-dNAP-1. Approximately 5% of the resulting immunoprecipitates were analyzed by Western blotting with antibodies against dNAP-1. Lanes: 1, purified dNAP-1 reference (150 ng); 2, immunoprecipitate obtained with preimmune serum; 3, immunoprecipitate obtained with anti-dNAP-1. (B) Coimmunoprecipitation (IP) of core histones from a crude, whole-embryo extract with antibodies against dNAP-1. A crude extract derived from ~300 mg of *Drosophila* embryos was subjected to immunoprecipitation with either preimmune serum or anti-dNAP-1. Approximately 25% of the resulting immunoprecipitates were analyzed by Western blotting with antibodies that recognize the core histones H2A, H2B, and H3, but not H4. Lanes: 1, purified core histone reference (40 ng of total protein); 2, immunoprecipitate obtained with preimmune serum; 3, immunoprecipitate obtained with anti-dNAP-1. Ig, immunoglobulin.

noprecipitation with antibodies against dNAP-1, all four core histones coimmunoprecipitated with dNAP-1 to the same extent (data not shown), and, hence, the coimmunoprecipitation procedure did not induce dissociation of H3 or H4 from dNAP-1. Therefore, the selectivity of the coimmunoprecipitation of H2A and H2B with dNAP-1 from the embryo extract appears to reflect the specific association of H2A and H2B with dNAP-1 in vivo. These results, when combined with the biochemical data described by Ishimi et al. (21, 23), suggest that NAP-1 binds to all four core histones, but with higher affinity to H2A and H2B than to H3 and H4. On the other hand, it is equally as plausible to consider the possibility that NAP-1 binds specifically to H2A and H2B in vivo and that other factors, such as N1 and/or CAF-1, function as chaperones for histones H3 and H4.

Localization of dNAP-1 in the *Drosophila* embryo. As an initial step in the characterization of dNAP-1 in vivo, we have examined the localization of dNAP-1 in the early *Drosophila* embryo. In other studies of the cellular localization of NAP-1, it has been reported to be in the nuclei of HeLa (human) cells (24) as well as primarily in the cytoplasm of yeasts (in this latter study, however, it was difficult to detect nuclear localization of yNAP-1 because of strong cytoplasmic staining [29]). Given these apparent differences, it was therefore additionally useful to investigate the localization of dNAP-1 in *Drosophila* embryos. Prior to describing these experiments, however, it is necessary to provide a brief description of some of the events that occur in the early embryo.

After fertilization of the *Drosophila* embryo, the somatic nuclei in the syncytial egg undergo 13 consecutive and nearly synchronous divisions in the absence of G_1 or G_2 phase (see, for instance, reference 15). Then, during the 14th cycle, cellularization of the nuclei occurs and gastrulation commences; and specific groups of cells, which have been designated mitotic domains, exhibit virtually invariable (from embryo-to-embryo) spatial and temporal characteristics with regard to the locations of these domains in the embryo and the timing of the onset of mitosis (which could alternatively be viewed as the duration of the G_2 period, which ranges from 30 to 130 min) of each of the domains (14). The mitotic domains have been designated by number in the temporal order in which each domain begins its 14th mitosis. Hence, mitotic domain 1 is the first to begin mitosis 14, whereas mitotic domain 25 is the last. Then, within 5 min of the conclusion of the 14th mitosis of each mitotic domain, the 15th S phase initiates and continues for about 35 to 45 min in all cells (13), and the spatial arrangement of the domains in S phase 15 is similar to that of mitosis 14. Moreover, because the 14th G_2 period can be as short as 30 min or as long as 130 min, an early mitotic domain could be in S or G_2 phase 15 while a late mitotic domain would simulta-

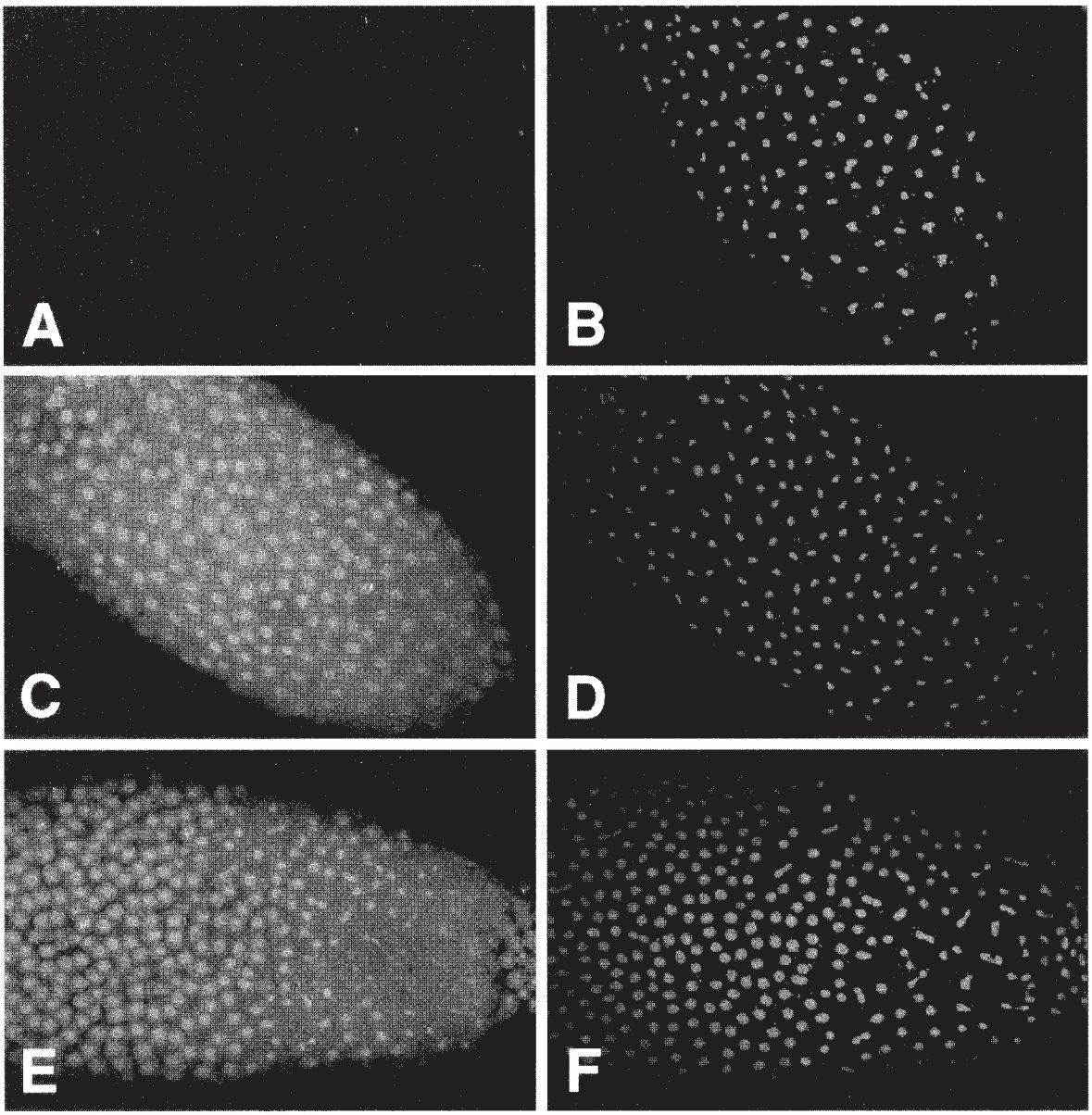


FIG. 7. Localization of dNAP-1 in the syncytial *Drosophila* embryo. Embryos were subjected to indirect immunofluorescence analysis with a monoclonal antibody (4A8 [22]) that recognizes dNAP-1. Propidium iodide (20 μg/ml) was used for DNA staining. (A) Control (background staining of a syncytial blastoderm with the secondary antibody only); (C) NAP-1 staining of a syncytial blastoderm (nuclear cycle 11, metaphase); (E) NAP-1 staining of a syncytial blastoderm (nuclear cycle 12, interphase); (B, D, and F) DNA staining of the embryos that correspond to those shown in panels A, C, and E, respectively. In panels C and E, there appears to be staining of NAP-1 in both the nuclei and cytoplasm, relative to that observed with the control (A).

neously be in G_2 or M phase 14. Lastly, there are also two domains of cells, denoted A and B, that contain cells that do not appear to undergo mitosis subsequent to the beginning of cycle 14 (14).

We thus examined the localization of dNAP-1 in *Drosophila* embryos by immunostaining and confocal microscopy. In the syncytial blastoderm prior to cycle 14, dNAP-1 is observed throughout the embryo with strong nuclear staining, which is seen both in S phase and in M phase (Fig. 7). (It also appears, upon close inspection of the mitotic nuclei, that dNAP-1 is excluded from the condensed chromatin during the metaphase.) Then, after the onset of cycle 14, the localization of dNAP-1 becomes more complex. Figure 8 shows serial planes of dNAP-1 staining in an embryo at approximately 75 to 80 min after the start of interphase 14. In this embryo, dNAP-1 is primarily in the cytoplasm of the cells in G_2 phase of cycle 14 (Fig. 8A, C, and E). (The majority of the cells that have not yet entered mitosis 14 should be in G_2 phase because S phase 14 lasts for approximately 35 to 45 min after the start of interphase 14 [13] and would be complete in this 75- to 80-min embryo.) In addition, in this embryo, the cells in the center of mitotic domain 1 are in early S phase 15, while those in the periphery of the domain are in telophase 14 (note that in mitotic domain 1, the first cells to enter mitosis 14 are near the center of the domain, from which a mitotic wave radiates to the periphery of the domain [14]). As shown in Fig. 8E and G, the cells in early S phase 15 exhibit strong nuclear staining of dNAP-1. In these cells, it appears that dNAP-1 is excluded

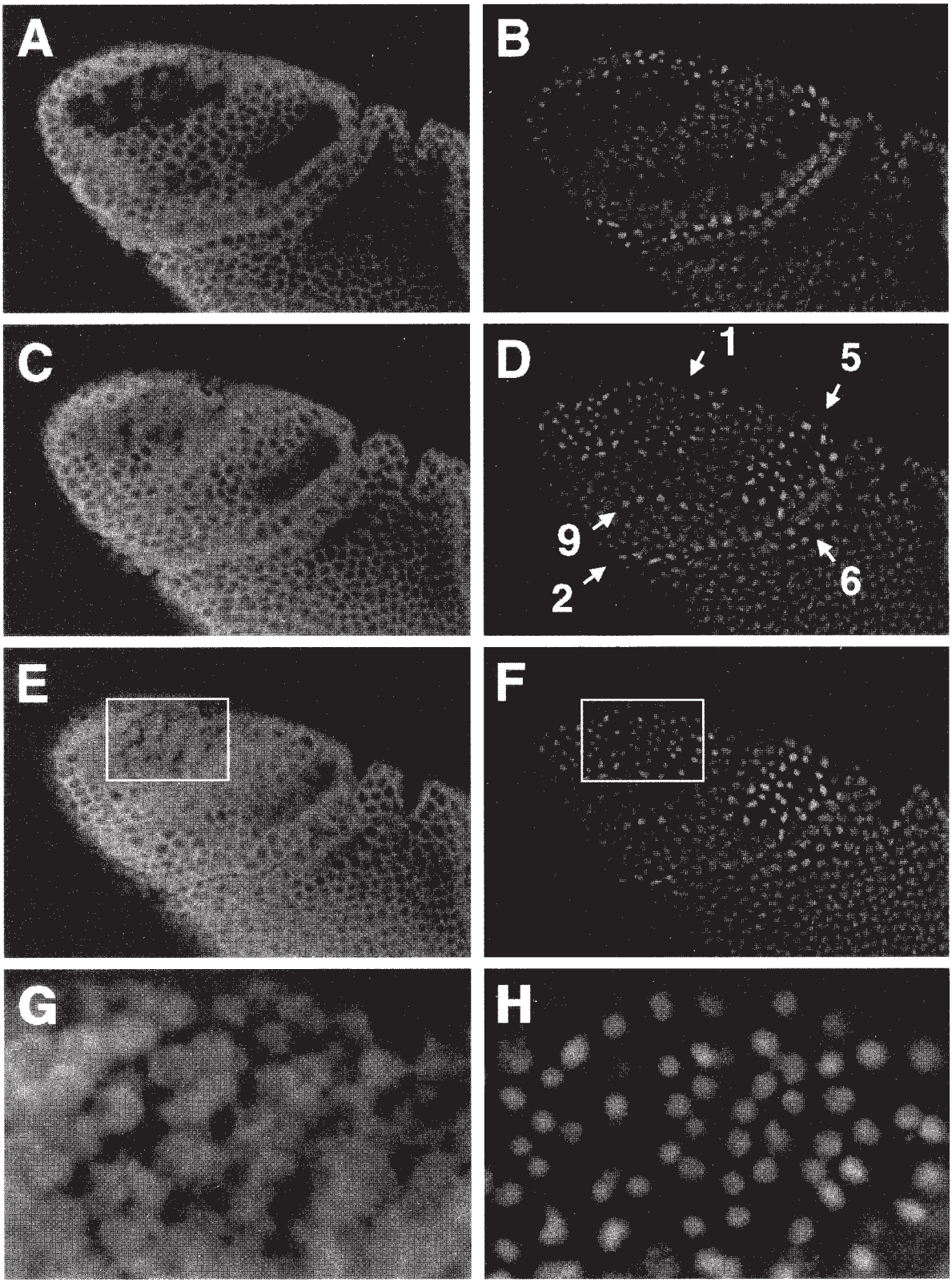


FIG. 8. Localization of dNAP-1 in the *Drosophila* embryo at approximately 75 to 80 min after the start of interphase 14. Different planes of the embryo were shown with the confocal microscope to illuminate the localization of the NAP-1 staining. The mitotic domains were designated according to the nomenclature described by Foe (14). (A, C, and E) NAP-1 staining in different planes of the same embryo, from the innermost plane (A) to that closest to the surface (E); (G) enlargement of the boxed region in panel E; (B, D, F, and H) DNA staining of the embryos that correspond to those shown in panels A, C, E, and G, respectively. The cells in the center of mitotic domain 1 are in early S phase (cycle 15), while those at the periphery of the domain are in telophase (cycle 14). The cells of mitotic domains 5 and 6 are at metaphase to telophase (cycle 14), and the cells of mitotic domain 9 are at prophase to metaphase (cycle 14).

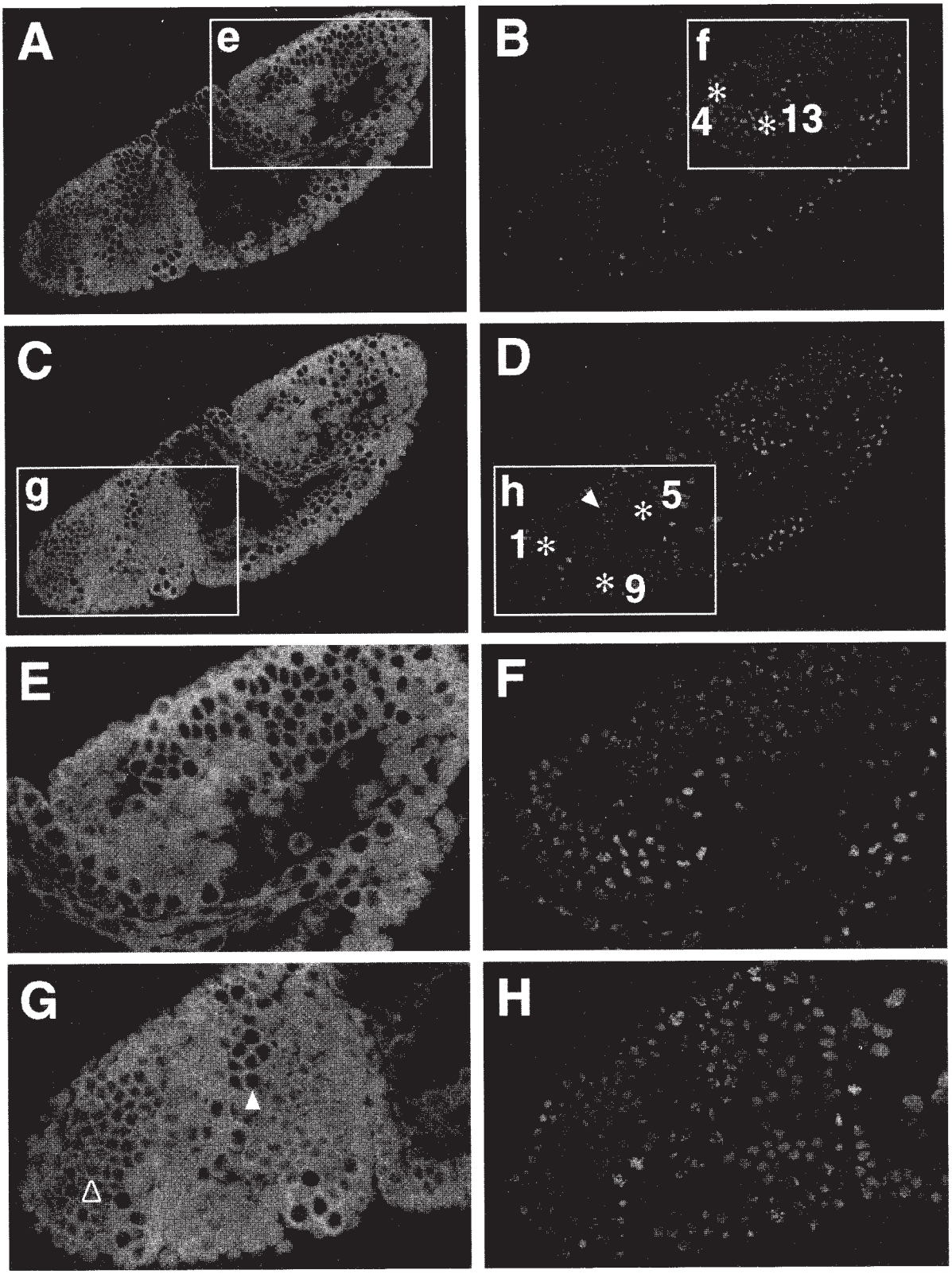


FIG. 9. Localization of dNAP-1 in the *Drosophila* embryo at approximately 90 min after the start of interphase 14. (A and C) NAP-1 staining in different planes of the same embryo, from the innermost plane (A) to that closer to the surface (C); (E and G) enlargement of the boxed region in panels A and C, respectively; (B, D, F, and H) DNA staining of the embryos that correspond to those shown in panels A, C, E, and G, respectively. Mitotic domain 13 is in metaphase (cycle 14), while mitotic domains 1, 4, 5, and 9 are in interphase (cycle 15). Domain B is indicated by a solid arrowhead. The open arrowhead in panel G depicts mitotic domain 1 (late S phase to early G_2 phase, cycle 15), while the solid arrowhead denotes domain B.

from the cytoplasm; however, this point is difficult to interpret because of the apparently larger size of the dNAP-1 staining (Fig. 8G) relative to DNA staining (Fig. 8H). (It is possible, for instance, that the staining of dNAP-1 both inside and immediately surrounding the nucleus is due to the transport of dNAP-1 from the cytoplasm [where it was localized during the G_2 phase of cycle 14] to the nucleus, whereby some of the dNAP-1 is in the nucleus while the remainder of the factor is in the vicinity of the nuclear envelope in the process of being translocated into the nucleus.) These data, therefore, suggest that dNAP-1 is localized to the nucleus during S phase, while it is mainly in the cytoplasm during G_2 phase.

We then determined the localization of dNAP-1 in a *Drosophila* embryo at approximately 90 min after the start of interphase 14 (Fig. 9). In this embryo, mitotic domain 1 is in late S or early G_2 phase, and there is weak nuclear staining and strong cytoplasmic staining of dNAP-1. In contrast, in mitotic domains 4, 5, and 9, which are in S phase, there is moderate-to-strong nuclear staining as well as cytoplasmic staining of dNAP-1. The results collectively lead to the conclusion that dNAP-1 is present in both the nucleus and the cytoplasm during S phase (as well as apparently enriched in the nucleus relative to the cytoplasm under some circumstances) and is primarily localized to the cytoplasm during G_2 phase as well as possibly late S phase. The finding that dNAP-1 becomes localized to the nucleus at the onset of S phase is consistent with its biochemical activity as a chromatin assembly factor. These data also provide a possible solution to the apparently contradictory reports of NAP-1 localization to the nuclei of HeLa cells (24) and to the cytoplasm of yeasts (29).

Summary and perspectives. In this work, we have described the cloning and analysis of dNAP-1, a core histone-binding protein that functions with partially purified activities in the dCAF-1 fraction in the ATP-facilitated assembly of regularly spaced nucleosomal arrays from purified core histones and DNA. The chromatin assembly reaction with these factors is highly efficient and, in the presence of histone H1, yields chromatin with a repeat length that is similar to that of native chromatin from *Drosophila* embryos. By coimmunoprecipitation analysis, dNAP-1 was found to be associated with histones H2A and H2B in a crude whole-embryo extract, which suggests that dNAP-1 is bound to the histones in vivo. Thus, while it has been commonly thought that NAP-1 functions independently to deposit nucleosomes randomly as a nonspecific histone transfer vehicle, these results suggest that NAP-1 is one component of a multifactor chromatin assembly machinery that mediates the ATP-facilitated assembly of regularly spaced nucleosomal arrays. In addition, studies of the localization of dNAP-1 in the *Drosophila* embryo revealed that the factor is present in the nucleus during S phase and is predominantly cytoplasmic during G_2 phase. On the basis of the ability of NAP-1 to bind to core histones, the localization of the factor in both the nucleus and the cytoplasm, and the function of NAP-1 in chromatin assembly, we suggest that NAP-1 acts as a core histone shuttle that delivers the histones from their site of synthesis in the cytoplasm to the chromatin assembly machinery in the nucleus (Fig. 10). Moreover, it has been observed that human CAF-1 colocalizes with DNA replication foci during S phase (34), and, thus, the nuclear localization of both NAP-1 and CAF-1 during S phase is consistent with the biochemical data that both factors are involved in chromatin assembly.

Somewhat unexpectedly, it was also found that the *S. cerevisiae* and *Xenopus* versions of NAP-1 bind either directly or indirectly (via $p34^{cdc2}$) to B-type cyclins (29, 30). In those studies, yNAP-1 was found to associate with Clb2, while *Xenopus* NAP-1 was observed to interact with cyclins B1 and B2. Then, on the basis of the analysis of yeast strains carrying various combinations of mutations in the genes encoding NAP-1 and mitotic cyclins, it was suggested that NAP-1 affects the function of cyclin B/$p34^{cdc2}$ (Clb2/$p34^{CDC28}$) kinase complexes. These results, combined with the biochemical data that purified NAP-1 did not yield the assembly of regularly spaced nucleosomal arrays, as seen for bulk chromatin in vivo, had led to some skepticism regarding the role of NAP-1 in chromatin assembly (29, 30). However, in contrast to earlier biochemical studies of NAP-1, our results indicate that NAP-1 functions in the ATP-facilitated assembly of regularly spaced nucleosomal arrays. These data, along with the coimmunoprecipitation of core histones with dNAP-1 from whole-embryo extracts (Fig. 6) and the localization of dNAP-1 in the nucleus during S phase (Fig. 7, 8, and 9), suggest that NAP-1 is a chromatin assembly factor. In our view, the possible dual function of NAP-1 in different processes at different phases of the cell cycle is both interesting and potentially significant.

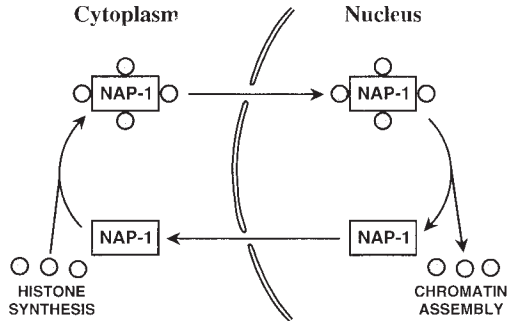


FIG. 10. Simple model for the function of NAP-1 as a core histone shuttle and chromatin assembly factor. Because NAP-1 appears to be multimeric, multiple histone species are shown to be associated with a NAP-1 protomer. An exact stoichiometry of histone-NAP-1 interaction is not meant to be implied.

Lastly, it seems likely that there are multiple factors, which include NAP-1, nucleoplasmin, CAF-1, and N1, that bind to core histones and participate in chromatin assembly. It is often thought that a key function of these factors might be to act as histone sheaths or chaperones that prevent nonspecific interaction of the highly charged histones with other biomolecules. However, there are two notable differences between NAP-1 and nucleoplasmin, both of which appear to bind preferentially to H2A and H2B relative to H3 and H4, which are as follows. First, NAP-1 has been found in eukaryotes from *S. cerevisiae* to *D. melanogaster* to humans, while nucleoplasmin has been identified only in *Xenopus* and *Drosophila* species (unpublished data), both of which appear to use nucleoplasmin to store maternally synthesized histones for chromatin assembly during early embryogenesis. (We have also examined the levels of dNAP-1 throughout *Drosophila* development by Western blot analysis, and the dNAP-1 protein was found to be present at comparable levels [as a fraction of the total protein] in embryos, larvae, pupae, and adults [53a]). Second, nucleoplasmin is localized exclusively in the nucleus (33, 39), whereas NAP-1 is present in both the nucleus and the cytoplasm. Thus, it appears more likely that NAP-1, rather than nucleoplasmin, might function as a core histone shuttle during cell growth.

ACKNOWLEDGMENTS

We thank Peter Gergen, Rohinton Kamakaka, Beth Blackwood, Jessica Tyler, Karen Robinson, and Catherine George for critical reading of the manuscript. We are most grateful to Theo Palmer for his invaluable guidance and help with confocal microscopy; Fred Gage for

generously allowing the use of the confocal microscope (supported by grant NINDS P01 NS28121); Victoria Foe, Peter Gergen, Jim Posakony, and Ethan Bier for generously sharing their expertise in the analysis of *Drosophila* embryos; Y. Ishimi and A. Kikuchi for monoclonal antibody 4A8 against NAP-1 and the yNAP-1 cDNA; Jessica Tyler, Todd Laverty, and Gerry Rubin for determination of the cytological location of the dNAP-1 gene; and Paul Kaufman, Bruce Stillman, and Rohinton Kamakaka for their advice and suggestions throughout the course of this work.

J.T.K. is a Presidential Faculty Fellow. This work was supported by grants from the National Institutes of Health, the National Science Foundation, and the Council for Tobacco Research to J.T.K. R.K. is supported by a grant (CA 13106) from the National Cancer Institute.

REFERENCES

1. **Banerjee, S., and C. R. Cantor.** 1990. Nucleosome assembly of simian virus 40 DNA in a mammalian cell extract. Mol. Cell. Biol. **10:**2863–2873.
2. **Becker, P. B., and C. Wu.** 1992. Cell-free system for assembly of transcriptionally repressed chromatin from *Drosophila* embryos. Mol. Cell. Biol. **12:**2241–2249.
3. **Blank, T. A., and P. B. Becker.** 1995. Electrostatic mechanism of nucleosome spacing. J. Mol. Biol. **252:**305–313.
4. **Boulikas, T.** 1993. Nuclear localization signals (NLS). Crit. Rev. Eukaryotic Gene Expr. **3:**193–227.
5. **Bulger, M., T. Ito, R. T. Kamakaka, and J. T. Kadonaga.** 1995. Assembly of regularly-spaced nucleosome arrays by dCAF-1 and a 56 kDa histone-binding protein. Proc. Natl. Acad. Sci. USA **92:**11726–11730.
6. **Bulger, M., and J. T. Kadonaga.** 1994. Biochemical reconstitution of chromatin with physiological nucleosome spacing. Methods Mol. Genet. **5:**241–262.
7. **Bürglin, T. R., I. W. Mattaj, D. D. Newmeyer, R. Zeller, and E. M. De Robertis.** 1987. Cloning of nucleoplasmin from *Xenopus laevis* oocytes and analysis of its developmental expression. Genes Dev. **1:**97–107.
8. **Croston, G. E., L. M. Lira, and J. T. Kadonaga.** 1991. A general method for the purification of H1 histones that are active for repression of basal RNA polymerase II transcription. Prot. Expr. Purif. **2:**162–169.
9. **Dilworth, S. M., and C. Dingwall.** 1988. Chromatin assembly in vitro and in vivo. BioEssays **9:**44–49.
10. **Dingwall, C., S. M. Dilworth, S. J. Black, S. E. Kearsey, L. S. Cox, and R. A. Laskey.** 1987. Nucleoplasmin cDNA sequence reveals polyglutamic acid tracts and a cluster of sequences homologous to putative nuclear localization signals. EMBO J. **6:**69–74.
11. **Dingwall, C., and R. A. Laskey.** 1991. Nuclear targeting sequences—a consensus? Trends Biochem. Sci. **16:**478–481.
12. **Drew, H. R.** 1993. Reconstitution of short-spaced chromatin from the histone octamer and either HMG-14,17 or histone H1. J. Mol. Biol. **230:**824–836.
13. **Edgar, B. A., and P. H. O'Farrell.** 1990. The three postblastoderm cell cycles of *Drosophila* embryogenesis are regulated in G_2 by string. Cell **62:**469–480.
14. **Foe, V. E.** 1989. Mitotic domains reveal early commitment of cells in *Drosophila* embryos. Development **107:**1–22.
15. **Foe, V. E., and B. M. Alberts.** 1983. Studies of nuclear and cytoplasmic behaviour during the five mitotic cycles that precede gastrulation in *Drosophila* embryogenesis. J. Cell Sci. **61:**31–70.
16. **Fujii-Nakata, T., Y. Ishimi, A. Okuda, and A. Kikuchi.** 1992. Functional analysis of nucleosome assembly protein, NAP-1: the negatively charged COOH-terminal region is not necessary for the intrinsic assembly activity. J. Biol. Chem. **267:**20980–20986.
17. **Gerace, L.** 1995. Nuclear export signals and the fast track to the cytoplasm. Cell **82:**341–344.
18. **Germond, J. E., B. Hirt, P. Oudet, M. Gross-Bellard, and P. Chambon.** 1975. Folding of the DNA double helix in chromatin-like structures from simian virus 40. Proc. Natl. Acad. Sci. USA **72:**1843–1847.
19. **Glikin, G. C., I. Ruberti, and A. Worcel.** 1984. Chromatin assembly in *Xenopus* oocytes: in vitro studies. Cell **37:**33–41.
20. **Gruss, C., and J. M. Sogo.** 1992. Chromatin replication. BioEssays **14:**1–8.
21. **Ishimi, Y., J. Hirosumi, W. Sato, K. Sugasawa, S. Yokota, F. Hanaoka, and M. Yamada.** 1984. Purification and initial characterization of a protein which facilitates assembly of nucleosome-like structure from mammalian cells. Eur. J. Biochem. **142:**431–439.
22. **Ishimi, Y., and A. Kikuchi.** 1991. Identification and molecular cloning of yeast homologue of nucleosome assembly protein I which facilitates nucleosome assembly in vitro. J. Biol. Chem. **266:**7025–7029.
23. **Ishimi, Y., M. Kojima, M. Yamada, and F. Hanaoka.** 1987. Binding mode of nucleosome-assembly protein (AP-I) and histones. Eur. J. Biochem. **162:**19–24.
24. **Ishimi, Y., W. Sato, M. Kojima, K. Sugasawa, F. Hanaoka, and M. Yamada.** 1985. Rapid purification of nucleosome assembly protein (AP-I) and production of monoclonal antibodies against it. Cell Struct. Funct. (Japan) **10:**373–382.
25. **Ishimi, Y., H. Yasuda, J. Hirosumi, F. Hanaoka, and M. Yamada.** 1983. A protein which facilitates assembly of nucleosome-like structures in vitro in mammalian cells. J. Biochem. **94:**735–744.
26. **Kamakaka, R. T., M. Bulger, and J. T. Kadonaga.** 1993. Potentiation of RNA polymerase II transcription by Gal4-VP16 during but not after DNA replication and chromatin assembly. Genes Dev. **7:**1779–1795.
27. **Kamakaka, R. T., M. Bulger, P. D. Kaufman, B. Stillman, and J. T. Kadonaga.** 1996. Postreplicative chromatin assembly by *Drosophila* and human chromatin assembly factor 1. Mol. Cell. Biol. **16:**810–817.
28. **Kaufman, P. D., R. Kobayashi, N. Kessler, and B. Stillman.** 1995. The p150 and p60 subunits of chromatin assembly factor I: a molecular link between newly synthesized histones and DNA replication. Cell **81:**1105–1114.
29. **Kellogg, D. R., A. Kikuchi, T. Fujii-Nakata, C. W. Turck, and A. W. Murray.** 1995. Members of the NAP/SET family of proteins interact specifically with B-type cyclins. J. Cell Biol. **130:**661–673.
30. **Kellogg, D. R., and A. W. Murray.** 1995. NAP1 acts with Clb2 to perform mitotic functions and to suppress polar bud growth in budding yeast. J. Cell Biol. **130:**675–685.
31. **Kim, U.-J., M. Han, P. Kayne, and M. Grunstein.** 1988. Effects of histone H4 depletion on the cell cycle and transcription of Saccharomyces cerevisiae. EMBO J. **7:**2211–2219.
32. **Kleinschmidt, J. A., A. Seiter, and H. Zentgraf.** 1990. Nucleosome assembly in vitro: separate histone transfer and synergistic interaction of native histone complexes purified from nuclei of *Xenopus laevis* oocytes. EMBO J. **9:**1309–1318.
33. **Krohne, G., and W. W. Franke.** 1980. A major soluble acidic protein located in nuclei of diverse vertebrate species. Exp. Cell Res. **129:**167–189.
34. **Krude, T.** 1995. Chromatin assembly factor 1 (CAF-1) colocalizes with replication foci in HeLa cell nuclei. Exp. Cell Res. **220:**304–311.
35. **Laskey, R. A., B. M. Honda, A. D. Mills, and J. T. Finch.** 1978. Nucleosomes are assembled by an acidic protein which binds histones and transfers them to DNA. Nature (London) **275:**416–420.

35a. **Laverty, T., and G. Rubin.** Unpublished data.

36. **Limbourg, B., and M. Zalokar.** 1973. Permeabilization of *Drosophila* eggs. Dev. Biol. **35:**382–387.
37. **McKnight, S. L., M. Bustin, and O. L. Miller, Jr.** 1978. Electron microscopic analysis of chromosome metabolism in the Drosophila melanogaster embryo. Cold Spring Harbor Symp. Quant. Biol. **42:**741–754.
38. **McKnight, S. L., and O. L. Miller, Jr.** 1977. Electron microscopic analysis of chromatin replication in the cellular blastoderm Drosophila melanogaster embryo. Cell **12:**795–804.
39. **Mills, A. D., R. A. Laskey, P. Black, and E. M. De Robertis.** 1980. An acidic protein which assembles nucleosomes in vitro is the most abundant protein in *Xenopus* oocyte nuclei. J. Mol. Biol. **139:**561–568.
40. **Nelson, T., R. Wiegand, and D. Brutlag.** 1981. Ribonucleic acid and other polyanions facilitate chromatin assembly in vitro. Biochemistry **20:**2594–2601.
41. **Noll, M., and R. D. Kornberg.** 1977. Action of micrococcal nuclease on chromatin and the location of histone H1. J. Mol. Biol. **109:**393–404.
42. **Sapp, M., and A. Worcel.** 1990. Purification and mechanism of action of a nucleosome assembly factor from *Xenopus* oocytes. J. Biol. Chem. **265:**9357–9365.
43. **Sealy, L., M. Cotten, and R. Chalkley.** 1986. *Xenopus* nucleoplasmin: egg vs. oocyte. Biochemistry **25:**3064–3072.
44. **Simon, H.-U., G. B. Mills, M. Kozlowski, D. Hogg, D. Branch, Y. Ishimi, and K. A. Siminovitch.** 1994. Molecular characterization of hNRP, a cDNA encoding a human nucleosome-assembly-protein-1-related gene product involved in the induction of cell proliferation. Biochem. J. **297:**389–397.
45. **Smith, P. A., V. Jackson, and R. Chalkley.** 1984. Two-stage maturation process for newly replicated chromatin. Biochemistry **23:**1576–1581.
46. **Smith, S., and B. Stillman.** 1991. Stepwise assembly of chromatin during DNA replication in vitro. EMBO J. **10:**971–980.
47. **Smith, S., and B. W. Stillman.** 1989. Purification and characterization of CAF-I, a human cell factor required for chromatin assembly during DNA replication in vitro. Cell **58:**15–25.
48. **Sogo, J. M., and R. A. Laskey.** 1995. Chromatin replication and assembly, p. 49–70. *In* S. C. R. Elgin (ed.), Chromatin structure and gene expression. IRL Press, New York.
49. **Stein, A., J. P. Whitlock, Jr., and M. Bina.** 1979. Acidic polypeptides can assemble both histones and chromatin in vitro at physiological ionic strength. Proc. Natl. Acad. Sci. USA **76:**5000–5004.
50. **Stillman, B.** 1986. Chromatin assembly during SV40 DNA replication in vitro. Cell **45:**555–565.
51. **Svaren, J., and R. Chalkley.** 1990. The structure and assembly of active chromatin. Trends Genet. **6:**52–56.
52. **Tremethick, D. J., and H. R. Drew.** 1993. High mobility group proteins 14 and 17 can space nucleosomes in vitro. J. Biol. Chem. **268:**11389–11393.
53. **Tremethick, D. J., and M. Frommer.** 1992. Partial purification, from *Xenopus laevis* oocytes, of an ATP-dependent activity required for nucleosome spacing in vitro. J. Biol. Chem. **267:**15041–15048.

53a. **Tyler, J. K., and J. T. Kadonaga.** Unpublished data.

54. **van Holde, K. E.** 1989. Chromatin. Springer-Verlag, New York.
55. **Varga-Weisz, P. D., T. A. Blank, and P. B. Becker.** 1995. Energy-dependent

chromatin accessibility and nucleosome mobility in a cell-free system. EMBO J. **14:**2209–2216.

56. **von Lindern, M., S. van Baal, J. Wiegant, A. Raap, A. Hagemeijer, and G. Grosveld.** 1992. *can*, a putative oncogene associated with myeloid leukemogenesis, may be activated by fusion of its 3′ half to different genes: characterization of the *set* gene. Mol. Cell. Biol. **12:**3346–3355.
57. **Walter, P. P., T. A. Owen-Hughes, J. Côté, and J. L. Workman.** 1995. Stimulation of transcription factor binding and histone displacement by nucleosome assembly protein 1 and nucleoplasmin requires disruption of the histone octamer. Mol. Cell. Biol. **15:**6178–6187.
58. **Wolffe, A. P.** 1995. Chromatin: structure and function. Academic Press, San Diego, Calif.
59. **Worcel, A., S. Han, and M. L. Wong.** 1978. Assembly of newly replicated chromatin. Cell **15:**969–977.
60. **Zucker, K., and A. Worcel.** 1990. The histone H3/H4 · N1 complex supplemented with histone H2A-H2B dimers and DNA topoisomerase I forms nucleosomes on circular DNA under physiological conditions. J. Biol. Chem. **265:**14487–14496.