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The p55 Subunit of *Drosophila* Chromatin Assembly Factor 1 Is Homologous to a Histone Deacetylase-Associated Protein

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To gain a better understanding of DNA replication-coupled chromatin assembly, we have isolated the cDNA encoding the smallest (apparent molecular mass, 55 kDa; termed p55) subunit of *Drosophila melanogaster* chromatin assembly factor 1 (dCAF-1), a multisubunit protein that is required for the assembly of nucleosomes onto newly replicated DNA in vitro. The p55 polypeptide comprises seven WD repeat motifs and is homologous to the mammalian RbAp48 protein, which is associated with the HD1 histone deacetylase. dCAF-1 was immunopurified by using affinity-purified antibodies against p55; the resulting dCAF-1 preparation possessed the four putative subunits of dCAF-1 (p180, p105, p75, and p55) and was active for DNA replication-coupled chromatin assembly. Moreover, dCAF-1 activity was specifically depleted with antibodies against p55. Thus, p55 is an integral component of dCAF-1. p55 is localized to the nucleus and is present throughout *Drosophila* development. Consistent with the homology between p55 and the HD1-associated RbAp48 protein, histone deacetylase activity was observed to coimmunoprecipitate specifically with p55 from a *Drosophila* nuclear extract. Furthermore, a fraction of the p55 protein becomes associated with the newly assembled chromatin following DNA replication. These findings collectively suggest that p55 may function as a link between DNA replication-coupled chromatin assembly and histone modification.

The assembly of newly replicated DNA into chromatin is a fundamental biological process that affects the structure and function of the genome (for reviews, see references 10, 18, 19, 22, 43, 45, and 49). In the analysis of chromatin assembly, it is important to consider not only the factors that mediate the formation of nucleosomes but also the nature of the components—the histones and DNA—from which chromatin is derived. With respect to the DNA, chromatin assembly appears to commence immediately after DNA replication, and nucleosomes are assembled onto the newly synthesized DNA. With regard to the histones, newly synthesized histones are modified (such as by acetylation and/or phosphorylation) and then deposited onto newly synthesized DNA, after which further modifications (such as deacetylation) can occur. In addition, roughly one-half of the histones in newly assembled chromatin are derived from the parental preexisting nucleosomes that are disassembled and reassembled during passage of the replication fork. Thus, in this manner, the process of nucleosome assembly is an important step in the creation of daughter chromosomes from the parental chromosome.

The biochemical analysis of DNA replication and chromatin assembly has led to the identification and characterization of a protein termed chromatin assembly factor 1 (CAF-1) from human cells (20, 37, 38, 42) and *Drosophila melanogaster* embryos (16). CAF-1 was identified by its ability to facilitate the assembly of newly replicated DNA into chromatin during T-antigen-mediated simian virus 40 (SV40) DNA replication. In these experiments, DNA replication-chromatin assembly reactions were performed with a cytosolic extract from 293 (human-derived) cells that contains all of the protein factors that are required for SV40 DNA replication, with the exception of T antigen, as well as the factors that are required for chromatin assembly, with the exception of CAF-1 (38, 42). Upon supplementation of the 293 cell extract with T antigen only, DNA replication but not chromatin assembly is observed, whereas upon the inclusion of both T antigen and CAF-1 in the reaction medium, both replication and chromatin assembly of the newly synthesized DNA are achieved. Interestingly, chromatin assembly with either human or *Drosophila* CAF-1 (hCAF-1 or dCAF-1, respectively) was found to occur preferentially but not obligatorily onto newly replicated DNA relative to bulk unreplicated DNA (16).

hCAF-1 consists of three subunits of 150, 60, and 50 kDa (38), whereas dCAF-1 appears to comprise four subunits of 180, 105, 75, and 55 kDa (16). The cDNAs encoding the 150- and 60-kDa subunits (designated p150 and p60) of hCAF-1 have been isolated, and the analysis of these proteins has led to the finding that p150, which contains an abundance of acidic amino acid residues, is a core histone-binding protein that exhibits a specificity for binding to newly synthesized H3 and H4, wherein the H4 is acetylated (20, 37). These data, combined with the knowledge that newly synthesized histones are acetylated in the cytoplasm prior to translocation to the nucleus and assembly into chromatin (1, 15, 30, 35, 39, 40), suggest that CAF-1 is a core histone chaperone that is involved in the deposition of newly synthesized H3 and H4. Thus, a model for the role of core histone chaperones in chromatin assembly is as follows: CAF-1 binds to newly synthesized H3 and (acetylated) H4 and mediates the formation of the H3-H4 tetramer onto newly replicated DNA; histones H2A-H2B are subsequently incorporated with the assistance of other histone chaperones, such as nucleoplasmin (5, 7, 21, 26, 36) or nucleosome assembly protein 1 (NAP-1 [11–14]), to give the complete histone octamer.

To study the function of CAF-1 in a metazoan whose growth

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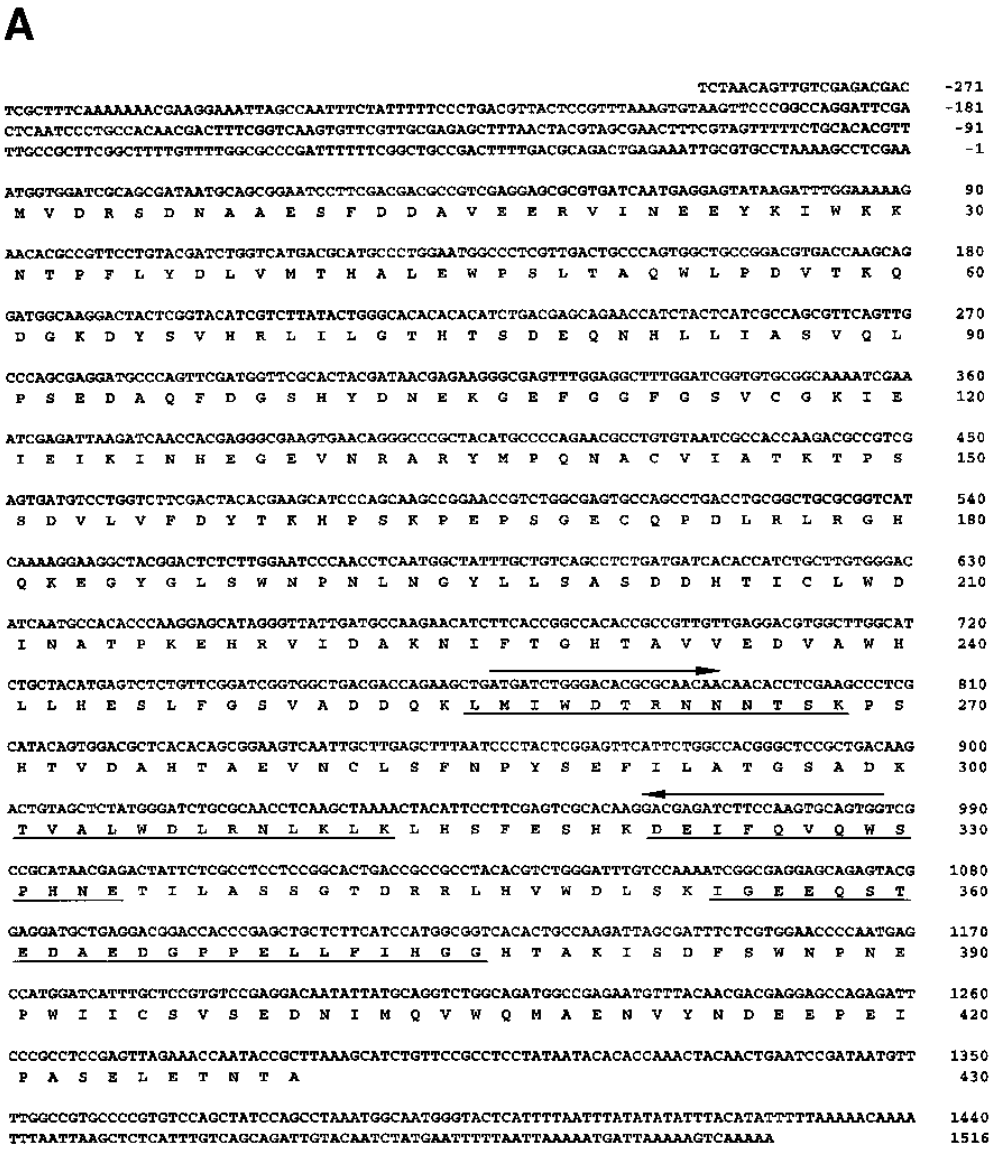

FIG. 1. The p55 subunit of dCAF-1 is a WD repeat-containing protein that is homologous to putative histone deacetylase-associated and Rb-binding proteins in mammals. (A) cDNA sequence and predicted amino acid sequence of p55. The longest open reading frame, as shown, encodes a protein of 430 amino acid residues with a calculated molecular mass of 48,604 Da. The arrows indicate the location of primers used to obtain the reverse transcription-PCR product. Sequences that were identified by peptide sequencing are underlined, and all four of the peptides that were sequenced from the native p55 protein are present in the predicted amino acid sequence. (B) Comparison of aligned amino acid sequences of *Drosophila* p55 with human RbAp48 (hRbAp48) (33) and RbAp46 (32), mouse RbAp48 (mRbAp48) and RbAp46 (32), and yeast Yel056p (GenBank accession number U18795). The complete sequences of all six proteins are shown. Amino acid residues that are identical in five or more of the six proteins are indicated by shading. The alignment was compiled by using the Genetic Computer Group Pile-up program; dots indicate the placement of gaps to maximize alignment between the sequences. (C) The WD repeat motifs in p55. In this alignment, ψ designates hydrophobic residues and ϕ denotes aromatic residues (41), as indicated by the gray shading. The structural triad of H, S/T, and D residues (indicated by *) (41) and the WD residues (denoted by °) of the WD repeat are highlighted in black.

and development can be readily analyzed by using both biochemical and genetic approaches, we have purified dCAF-1 from *Drosophila* embryos and tentatively identified its constituent polypeptides (16). In this report, we describe the cloning and characterization of the 55-kDa subunit of dCAF-1, termed p55. We have found that p55 is an integral subunit of dCAF-1 and is a WD repeat-containing protein that is homologous to a mammalian factor, termed RbAp48, which is associated with the HD1 histone deacetylase (44). These data suggest that p55 may function as a link between DNA replication, chromatin assembly, and histone modification.

MATERIALS AND METHODS

Purification and protein sequencing of dCAF-1 p55. dCAF-1 was purified from *Drosophila* embryos (collected 0 to 6 h after egg deposition) as described previously (16). dCAF-1 from the glycerol gradient peak was subjected to electrophoresis in a sodium dodecyl sulfate (SDS)–7.5% polyacrylamide gel and stained with Coomassie brilliant blue G (Sigma). The p55 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).

Cloning of p55. Degenerate primers that correspond to the expected coding sequence (including preferred *Drosophila* codon usage [3]) of the p55 peptides were synthesized. Reverse transcription-PCRs with one pair of primers (indicat-

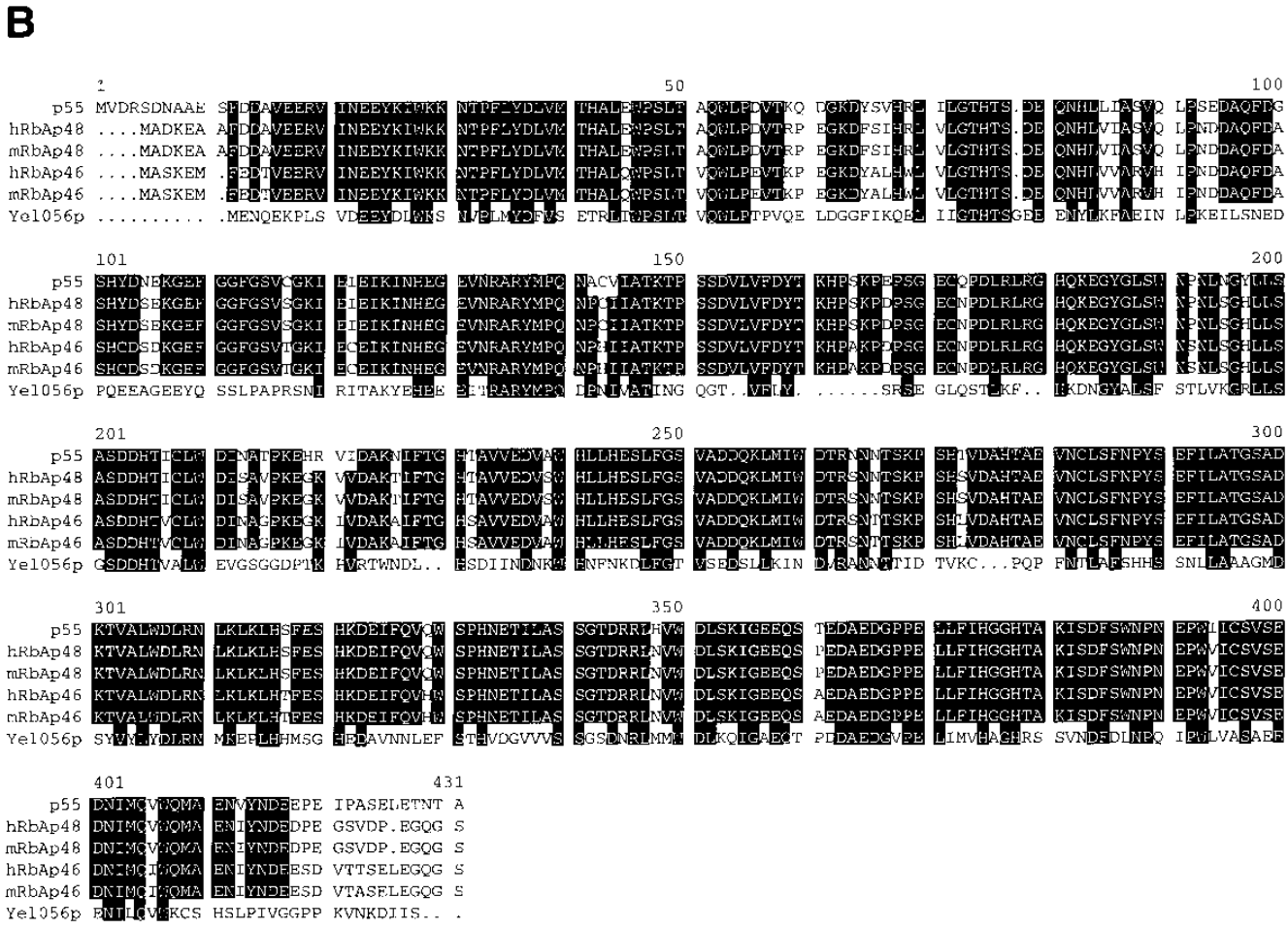

FIG. 1—*Continued.*

ed in Fig. 1A) and *Drosophila* embryo mRNA yielded a single discrete product of about 230 bp, and a second round of PCR with nested primers suggested that this product was likely to be a segment of the p55 cDNA. DNA sequencing confirmed that this cDNA fragment encoded p55 peptide sequences that were not included in the primers. Fifteen independent cDNAs were isolated by screening a *Drosophila* embryo cDNA library (0- to 4-h embryos) in λZAPII (Stratagene) with the radiolabeled, PCR-amplified cDNA fragment. The entire cDNA sequences of both DNA strands of three independent cDNA isolates were sequenced. These p55 cDNAs appear to contain the complete coding sequence because bacterial expression of the p55 open reading frame yielded a protein of approximately 55 kDa that comigrated with the native p55 polypeptide in dCAF-1 by SDS-polyacrylamide gel electrophoresis. The 15 cDNA clones fell into two distinct groups. Seven cDNAs differed from the other eight cDNAs by two silent nucleotide changes within the open reading frame and two single-nucleotide differences in the 3′ untranslated sequence. Because genomic Southern blots indicated that the p55 gene is a single-copy gene (data not shown), we assume that these sequence differences between the two groups of p55 clones reflect a genetic polymorphism within the *Drosophila* population that was used to construct the cDNA library. The p55 coding region was PCR amplified and subcloned into the *Nde*I and *Bam*HI sites of pET3c (Novagen) to give the bacterial expression plasmid pETp55, and the coding region was completely resequenced to confirm the absence of mutagenesis during the PCR process.

Generation of recombinant p55 protein and antisera. p55 protein synthesized with plasmid pETp55 in *Escherichia coli* was extracted from inclusion bodies with 6 M guanidine-HCl. The p55 protein was further purified to >95% homogeneity by successive chromatographic steps on Q-Sepharose FF, SP-Sepharose HP, Mono-Q, and DEAE-Sepharose FF resins (Pharmacia). Rabbits were immunized with the purified recombinant p55 protein to give anti-p55 antisera. Antibodies against p55 were affinity purified from the antisera by column chromatography with a p55 affinity resin (prepared by coupling of purified recombinant p55 to Affi-Prep 10 activated resin [Bio-Rad]). The antibodies were eluted from the resin with 100 mM glycine-HCl (pH 2.0). The resulting solution was neu-

tralized with 1/4 volume of 1 M Tris-HCl (pH 8.0), and then the affinity-purified antibodies were dialyzed into phosphate-buffered saline (3.4 mM sodium phosphate [dibasic], 1.5 mM potassium phosphate [monobasic], 137 mM NaCl, 2.7 mM KCl). All protein concentrations were determined with the bicinchoninic acid protein assay (Pierce) by using bovine serum albumin as the reference.

SDS-polyacrylamide gel electrophoresis and Western blot (immunoblot) analysis. Protein samples were subjected to electrophoresis on SDS–7.5% polyacrylamide gels. Proteins were either stained with Coomassie brilliant blue R-250 or transferred to nitrocellulose membranes. Western blot analyses used rabbit polyclonal antibodies (anti-p55 antibodies and affinity-purified biotin-conjugated anti-p55 antibodies) or mouse antibodies (anti-α-tubulin 3A5 [31]). The proteins were detected with the following reagents, as indicated for each blot: ^{125}I-labeled protein A (ICN); ABC reagents (Vector) in conjunction with the biotin-coupled antibodies; enhanced chemiluminescence (Amersham) in conjunction with horseradish peroxidase-coupled second antibodies; or 5-bromo-4-chloro-3-indolyl-phosphate and nitroblue tetrazolium (BCIP-NBT) in conjunction with alkaline phosphatase-coupled second antibodies. All experiments in this study were performed a minimum of two times to ensure reproducibility of the data.

SV40 DNA replication-chromatin assembly assay. SV40 DNA replication reactions with CAF-1-deficient human 293 cell cytosolic extracts, SV40 large T antigen, pSV011 plasmid DNA, and [α-^{32}P]dATP were performed and analyzed as described previously (38, 42), with the specific modifications reported by Kamakaka et al. (17). CAF-1-dependent assembly of nucleosomes during the replication reaction was observed by the incorporation of negative supercoils into the newly replicated DNA, as detected by autoradiography.

Immunoprecipitation of CAF-1 activity. Partially purified dCAF-1 (300 μl; purified from a *Drosophila* embryo nuclear extract through the SP-Sepharose FF, Q-Sepharose FF, and hydroxylapatite columns as described by Kamakaka et al. [16]) was incubated with 20 μg of either affinity-purified anti-p55 or the corresponding preimmune sera in buffer A (25 mM Tris-HCl [pH 7.5], 1 mM EDTA, 0.1% [vol/vol] Nonidet P-40, 10% [vol/vol] glycerol) containing 100 mM NaCl for 1 h at 4°C. Protein A–Sepharose CL-4B (30 μl of a 1:1 [vol/vol] slurry of protein A-Sepharose [Pharmacia]–buffer A containing 100 mM NaCl) was added, and the mixture was incubated for an additional 1 h. The beads were then washed extensively in buffer A (containing 100 mM NaCl, or as indicated) prior to further characterization.

Developmental Western blots. Protein extracts were prepared from staged wild-type (Canton S) *Drosophila* embryos, larvae, pupae, and adult flies as follows. Approximately 100 embryos (or equivalent mass of other stages) were frozen in liquid nitrogen and pulverized by using a small plastic homogenizer (catalog no. 749520-0000; Kontes) on ice. Next, homogenization buffer (50 μl; 10 mM Tris-HCl [pH 7.5], 60 mM NaCl, 10 mM EDTA, 0.15 mM spermine, 0.15 mM spermidine) was added, and homogenization was continued for an additional 1 min on ice. The extracts were cleared by centrifugation for 1 min in a microcentrifuge, and aliquots were removed for protein concentration determination. Then 1 volume of electrophoresis sample buffer containing 2% SDS and 1.4 M 2-mercaptoethanol was added to each sample, which was then boiled for 5 min. For each developmental stage, 50 μg of total protein was used in a single gel lane of the Western blot analysis.

Embryo staining. *Drosophila* Canton S or Stg [4] (8) embryos were fixed and stained by using procedures similar to those previously described (14). In these experiments, affinity-purified antibodies against p55 were used.

Sucrose gradient sedimentation analysis. SV40 DNA replication-chromatin assembly reactions were performed, as described above, at 10 times the normal scale. After completion, the reaction mixtures were layered onto 20 to 40% (wt/vol) sucrose gradients in buffer A (plus 150 mM NaCl, 0.2 mM phenylmethylsulfonyl fluoride and 0.5 mM dithiothreitol) (the gradients were prepared simultaneously in centrifuge tubes by using a six-channel device; the linearity of the sucrose gradients as well as the consistency of the sucrose concentrations in the corresponding fractions obtained from each of the tubes were confirmed by measurement of the refractive index of the gradient fractions), and the gradients were subjected to centrifugation in a Beckman SW41 rotor at 30,000 rpm for 23 h at 4°C. Following centrifugation, the gradients were collected in 16 fractions from top to bottom. Aliquots of each fraction were treated with 20 mM EDTA and proteinase K. The resulting DNA was precipitated and analyzed by 1% agarose gel electrophoresis. The newly replicated DNA was detected by autoradiography. In parallel, aliquots of each of the same sucrose gradient fractions were also analyzed by (i) Western blotting with the p55 antibodies and (ii) Southern blotting with ^{32}P-labeled DNA that is complementary to the plasmid DNA used in the replication reactions (to detect DNA that is related to the exogenously added DNA).

Histone deacetylase assay. A crude nuclear extract (4 ml; a 0.4 M NaCl nuclear extract was subjected to precipitation with ammonium sulfate [25 to 60% saturation] and then dialyzed into buffer A containing 150 mM NaCl, 0.2 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol) from *Drosophila* embryos (collected 0 to 12 h after egg deposition) was incubated with protein A-Sepharose (100 μl of a 1:1 [vol/vol] slurry; Pharmacia Biotechnology) for 2 h at 4°C, and then the protein A-Sepharose beads were removed by microcentrifugation (20 s at 2,000 rpm) to deplete components in the extract that bound nonspecifically to the protein A-Sepharose resin. The resulting supernatant (1 ml, for each subsequent incubation) was combined with affinity-purified anti-p55 antibody (10 μg), the corresponding preimmune serum (5 μl), or a control antiserum against a *Drosophila* protein of approximately 200 kDa (5 μl), and the mixtures were incubated at 4°C for 16 h. Next, to each sample, protein A-Sepharose (15 μl of a 1:1 [vol/vol] slurry in buffer A containing 150 mM NaCl) was added, and the mixtures were incubated at 4°C for 1 h. The pellets were then washed extensively (each sample was washed six times with 1 ml per wash) with buffer A containing 500 mM NaCl. Lastly, the pellets were washed (one time with 0.2 ml) with HDA buffer (75 mM Tris-HCl [pH 6.8] containing 275 mM NaCl, 0.1 mM EDTA, and 2 mM 2-mercaptoethanol). Histone deacetylase activity was measured by the release of [^{3}H]acetate from [^{3}H]acetyllysine histones (from HeLa cells; generous gift of A. Carmen and M. Grunstein, University of California, Los Angeles) as described by Carmen et al. (4).

Analysis programs. The National Center for Biotechnology Information BLAST and RETRIEVE electronic mail servers were used to identify and to obtain sequences related to those of p55 (2). Alignments were performed by using the Genetics Computer Group Pile-up program.

Nucleotide sequence accession number. The p55 sequence has been deposited in GenBank under accession number U62388.

RESULTS

Isolation of the cDNA encoding dCAF-1 p55. To study the role of dCAF-1 in chromatin assembly, we have isolated the cDNA encoding the smallest subunit (apparent molecular mass of 55 kDa), which we term p55. To this end, we purified dCAF-1 from *D. melanogaster* embryos, isolated the p55 dCAF-1 subunit by SDS-polyacrylamide gel electrophoresis, and subjected the protein to proteolytic digestion and amino acid sequence analysis. On the basis of this partial amino acid sequence data, we used PCR to isolate 15 independent cDNAs encoding p55. Northern (RNA) blot analysis with poly(A)$^+$ RNA from *Drosophila* embryos (collected 0 to 12 h after egg deposition) identified a single p55 transcript of 2.1 kb (data not shown), which is slightly longer than the cDNA of 1,807 nucleotides (Fig. 1A). Southern blot analysis with *Drosophila* genomic DNA revealed that the gene encoding p55 appears to be present in a single copy (data not shown). By in situ hybridization to polytene chromosomes, the p55 locus was mapped to region 88E, bands 8 and 9, on the right arm of the third chromosome (27).

dCAF-1 p55 is a WD repeat protein that is homologous to mammalian histone deacetylase-associated and Rb-binding proteins. Examination of the predicted amino acid sequence of p55 (Fig. 1A) revealed that it is a *Drosophila* homolog of two mammalian proteins termed RbAp46 and RbAp48 (Fig. 1B), which were originally identified by their ability to bind to a fragment of retinoblastoma protein (Rb) in vitro (32, 33). RbAp48 was also found to be associated with the HD1 histone deacetylase (44). There is an estimated 87% amino acid identity between *Drosophila* p55 and either mouse or human RbAp48, while there is ~84% identity between p55 and either mouse or human RbAp46 (Fig. 1B). Moreover, p55 appears to be related to two proteins in *Saccharomyces cerevisiae*: a protein (as deduced from an open reading frame) designated Yel056p (~34% identity [Fig. 1B]) and the protein encoded by the *MSI1* gene (34) (~26% identity). Thus, p55 appears to be conserved from *S. cerevisiae* to humans.

p55 is a member of the WD repeat family of proteins (Fig. 1C) (for reviews, see references 28 and 29). Members of this functionally diverse group of proteins have 4 to 10 copies of the WD repeat, which is a motif of approximately 40 amino acid residues that includes a conserved Trp-Asp dipeptide, the WD of the repeated sequence. The structures of the WD repeat motifs in two different G-protein β subunits have been solved (25, 41, 48). These studies revealed that the seven WD repeats in the G-protein β subunit form a β-propeller structure, wherein each of the seven "blades" of the propeller contains the residues of one WD repeat unit (though the beginning and end of each blade does not coincide with the beginning and end of the WD repeat sequence as typically written with the

WD at the C-terminal end; see, for example, Fig. 1C). The structural analysis further suggested the potential contribution of other conserved residues, such as a His, Ser/Thr, and Asp (not the same Asp in the WD) structural triad, in the formation of the β-propeller. Notably, the His, Ser/Thr, and Asp residues of this triad as well as other conserved hydrophobic residues that are thought to be important for the formation of the β-propeller (41) are present in many of the seven WD repeats of p55 (Fig. 1C). Hence, given the common features of the WD repeats in p55 and G-protein β subunits, it is likely that p55 also exists as a β-propeller.

p55 is an integral component of dCAF-1. The assignment of p55 as a subunit of dCAF-1 has been based on the copurification of p55 and three other polypeptides (p75, p105, and p180) with dCAF-1 activity (16), and it was therefore important to test more thoroughly whether p55 is a component of dCAF-1. First, by using antibodies against p55, we compared the migration of p55 protein with that of dCAF-1 activity upon glycerol gradient sedimentation of partially purified dCAF-1 (purified by ammonium sulfate fractionation and six chromatographic steps as described by Kamakaka et al. [16]). As shown in Fig. 2, the presence of p55 protein, as determined by Western blotting (Fig. 2B), correlated with dCAF-1 activity, as determined by the DNA replication-chromatin assembly assay (Fig. 2A). Thus, p55 comigrates with dCAF-1 activity during glycerol gradient sedimentation.

Next, we tested whether p55 is associated with the other subunits of dCAF-1 by coimmunoprecipitation analysis (Fig. 3). In these experiments, a partially purified dCAF-1 fraction (purified through three chromatographic steps) was immunoprecipitated with affinity-purified anti-p55 antibodies, and the resulting precipitate and unbound material fractions were characterized. Where noted, as an additional control, we had preincubated the anti-p55 antibodies with excess purified recombinant p55 protein to inhibit the binding of the antibodies to the native p55 in the dCAF-1 fraction. As shown in Fig. 3A, native p55 was specifically immunoprecipitated with the anti-p55 antibodies as well as depleted from the supernatant fraction. To determine the protein composition of the anti-p55 immunoprecipitate, we subjected the samples to SDS-polyacrylamide gel electrophoresis and staining with Coomassie blue (Fig. 3B). This experiment revealed that proteins of 180, 105, and 75 kDa (which comigrate with the p180, p105, and p75 subunits of dCAF-1 that is purified by conventional methods) were the major constituents of the immunoprecipitate (aside from p55 and immunoglobulins) after the beads were washed with buffer containing 1 M NaCl (Fig. 3B, lane 4). In addition, p180, p105, and p75 were not coimmunoprecipitated either with preimmune sera or with the blocked anti-p55 antibodies (Fig. 3B, lanes 2 and 5). Hence, by comparison of the partially purified dCAF-1 that was used as the starting material (Fig. 3B, lane 1) with the anti-p55 immunoprecipitates (Fig. 3B, lanes 3 and 4), it is evident that the immunoprecipitation of p55 results in the specific purification of dCAF-1.

To confirm further the identity of the anti-p55 immunoprecipitate as dCAF-1, we directly measured the dCAF-1 activity of each of the samples in the immunoprecipitation experiment by using the SV40 DNA replication-chromatin assembly assay (Fig. 3C). dCAF-1 activity coprecipitated with the anti-p55 antibodies but not with the preimmune sera or with the blocked anti-p55 antibodies (Fig. 3C, lanes 3 to 6). In addition, dCAF-1 activity was specifically depleted from the anti-p55 supernatants. These data collectively indicate that p55 is an integral subunit of dCAF-1. Moreover, because we have observed the same polypeptides (p180, p105, p75, and p55) in dCAF-1 that has been purified either by conventional methodology (16) or by immunoprecipitation (Fig. 3), we suggest that these four polypeptides are subunits of dCAF-1.

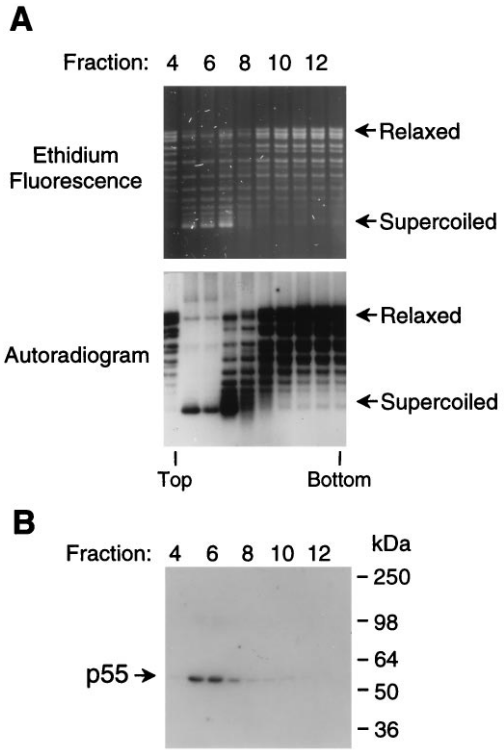

FIG. 2. The p55 protein copurifies with CAF-1 activity. dCAF-1 was partially purified from *Drosophila* embryos and then subjected to 15 to 50% (vol/vol) glycerol gradient sedimentation. (A) Determination of CAF-1 activity by using the SV40 DNA replication-chromatin assembly assay. Replication reactions were performed with 9 μl of each glycerol gradient fraction. The newly replicated DNA species were resolved by electrophoresis in a 1% (wt/vol) agarose gel and detected by autoradiography of the dried gel (the newly replicated DNA was radiolabeled by the inclusion of [α-^{32}P]dATP in the replication reaction medium). 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 and supercoiled DNA species are indicated. (B) Western blot analysis of the sedimentation of p55. Aliquots of each of the identical glycerol gradient fractions that were used in panel A were subjected to trichloroacetic acid precipitation, and the proteins were resolved by SDS–7.5% polyacrylamide gel electrophoresis followed by Western blot analysis. The blot was probed with anti-p55 antibodies, and p55 was detected with ^{125}I-labeled protein A and autoradiography.

dCAF-1 p55 is a predominantly nuclear protein that is present throughout *Drosophila* development. We then examined the subcellular localization of p55 in *Drosophila* embryos. By indirect immunofluorescence analysis with affinity-purified antibodies, p55 was observed mainly in the nuclei of syncytial blastoderm embryos (nuclear cycle 13) at either S phase or M phase (Fig. 4C and E). Figure 4G reveals the localization of p55 in an embryo at approximately 75 min after the start of interphase 14. As described by Foe (9), specific regions of this embryo, termed mitotic domains, are in different stages of the cell cycle, and mitotic domains 1 and 5 are in M phase of cycle 14, while domains 18, 20, and B are in G_2 phase of cycle 14. Interestingly, as seen in Fig. 4G, p55 appears to be distributed throughout the entire cell during mitosis (see mitotic domains 1 and 5), while the protein is in the nucleus of the cells in G_2 phase (see domains 18, 20, and B). Thus, in embryos prior to cellularization, p55 is mainly in the nuclei during S phase and M phase, whereas after cellularization, p55 appears to be nuclear except during M phase, in which it is present throughout

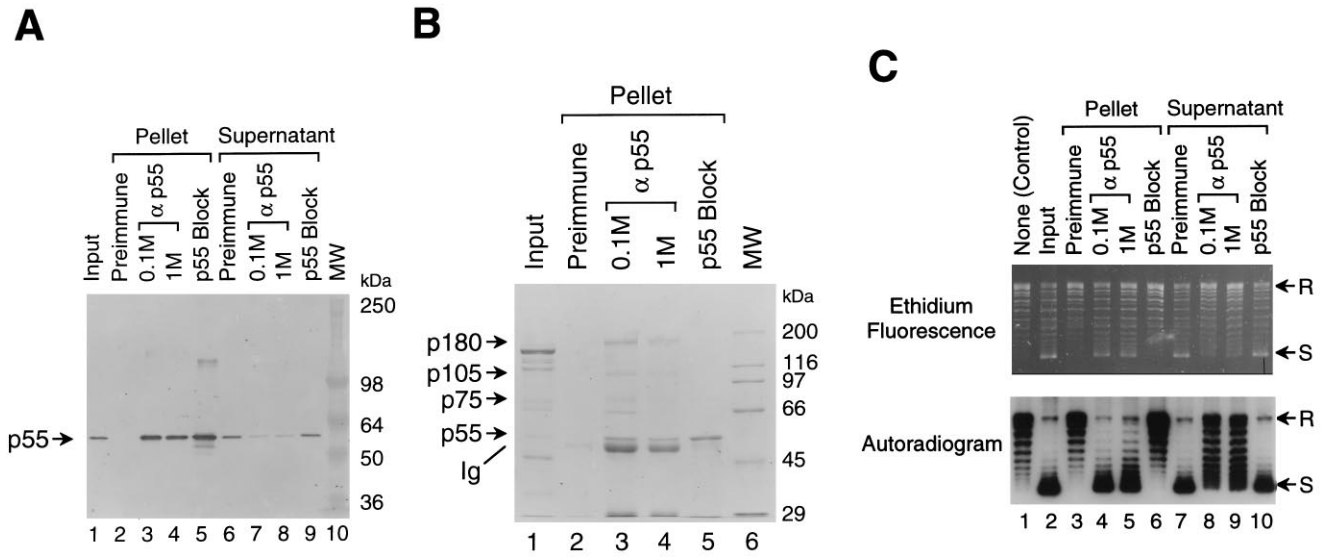

FIG. 3. Immunopurification of dCAF-1 with anti-p55 antibodies. Immunoprecipitation reactions were performed by using affinity-purified anti-p55 antibodies (α p55) or the corresponding preimmune serum as a control. The input sample is the partially purified dCAF-1-containing fraction that was used as a starting material in the immunoprecipitation analysis. p55 Block indicates that the anti-p55 antibodies were blocked by preincubation with excess purified recombinant p55 protein; 0.1 M and 1 M indicate that the immunoprecipitates were washed with buffer containing either 0.1 or 1 M NaCl. To permit a direct comparison of the results, the data shown were obtained with the identical protein samples. (A) Western blot analysis of the immunoprecipitation reactions. The blot was probed with biotin-conjugated antibodies against p55, which was detected with BCIP-NBT. MW, molecular weight markers. (B) Analysis of the immunoprecipitates by SDS-polyacrylamide gel electrophoresis and staining with Coomassie blue. Aliquots of the immobilized protein-antibody-bead complexes (Pellet) as well as the sample prior to immunoprecipitation (Input) were subjected to SDS–7.5% polyacrylamide gel electrophoresis and staining with Coomassie brilliant blue R-250. The apparent molecular masses of the proteins that are immunoprecipitated by the anti-p55 antibodies are 180, 105, 75, and 55 kDa. The polypeptide of approximately 65 kDa in lane 3 is not typically observed in these immunoprecipitation experiments. The p55 polypeptide that can be seen in lane 5 is the recombinant p55 that was used to block the binding of the endogenous p55 to the antibodies. (C) Determination of CAF-1 activity in the immunoprecipitates. CAF-1 activity was measured by using the SV40 DNA replication-chromatin assembly assay under standard conditions. Lane 1, no sample added (negative control); lane 2, the input fraction (1 μl); lanes 3 to 6, immobilized protein complexes (1 μl/20-μl total volume); and lanes 7 to 10, corresponding supernatants of the immunoprecipitated samples in lanes 3 to 6 (1 μl/350-μl total volume). 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.

the cell, possibly as a consequence of the partial breakdown of the nuclear envelope during mitosis in *D. melanogaster.*

Whole-mount immunostaining of *Drosophila* embryos further revealed that p55 is present in apparently all nuclei of pre- and postblastoderm embryos (Fig. 5A and B). Interestingly, as shown in Fig. 5B, the large cells of the amnioserosa (indicated by the arrow) had already undergone their last cell division, yet p55 was still in the nuclei of these cells. In addition, as depicted in Fig. 5C, p55 is localized to the nuclei of G_2-phase arrested cells of *string* mutant embryos. It therefore appears that p55 is a predominantly nuclear protein, which is consistent with the proposed role of p55 in chromatin assembly and the previous description of the mammalian homologs of p55, RbAp46 and RbAp48, as nuclear proteins (32, 33).

To determine whether the levels of p55 vary in the course of the growth and development of the organism, we carried out Western blot analysis of proteins derived from different developmental stages of *D. melanogaster*. As shown in Fig. 6, the level of p55 (relative to an α-tubulin reference, which correlates with the total protein) is highest during early embryogenesis and then decreases by a factor of approximately 10 in larvae, pupae, and adults. The presence of the highest levels of p55 in early embryos and at lower yet readily detectable amounts during later stages of development is consistent with the involvement of p55 in the process of DNA replication-coupled chromatin assembly, which occurs throughout the life cycle but at particularly high levels in the early embryo, wherein the nuclei are dividing approximately every 10 min.

We also estimated the amount of p55 protein in the embryo by quantitative Western blot analysis by using the purified recombinant protein as a standard. In this experiment, we determined that 25 embryos (at 3 to 6 h after egg deposition) contained approximately 5.3 ng of p55 (data not shown). This amount of protein corresponds to about 220,000 molecules per nucleus, based on an estimated average of 12,000 nuclei per embryo. Thus, the data collectively indicate that p55 is a relatively abundant nuclear protein that is present at substantial levels throughout *Drosophila* development.

Association of p55 with newly assembled chromatin. To address the mechanism of dCAF-1-mediated chromatin assembly and the involvement of p55 in this process, we examined whether p55 associates with chromatin that is assembled following DNA replication. We performed SV40 DNA replication-chromatin assembly reactions in the presence or the absence of dCAF-1 (or in absence of SV40 T antigen, which is required for DNA replication, as a control) and separated the components of the reaction mixture by sucrose gradient sedimentation. The total DNA species that were derived from the SV40 origin-containing plasmid that was used as the DNA template were detected by Southern blot analysis of each of the gradient fractions (Fig. 7, upper panels). The newly replicated DNA in the gradient fractions was detected by agarose gel electrophoresis and autoradiography (Fig. 7, middle panels), while the presence of p55 in the same fractions was determined by Western blot analysis (Fig. 7, lower panels). Comparison of reactions that were carried out in either the presence or the absence of dCAF-1 (Fig. 7A and B) shows that chromatin assembly was dependent upon dCAF-1 and that the packaging of the newly replicated DNA into chromatin resulted in an increase in the sedimentation of the DNA from fractions 4 to

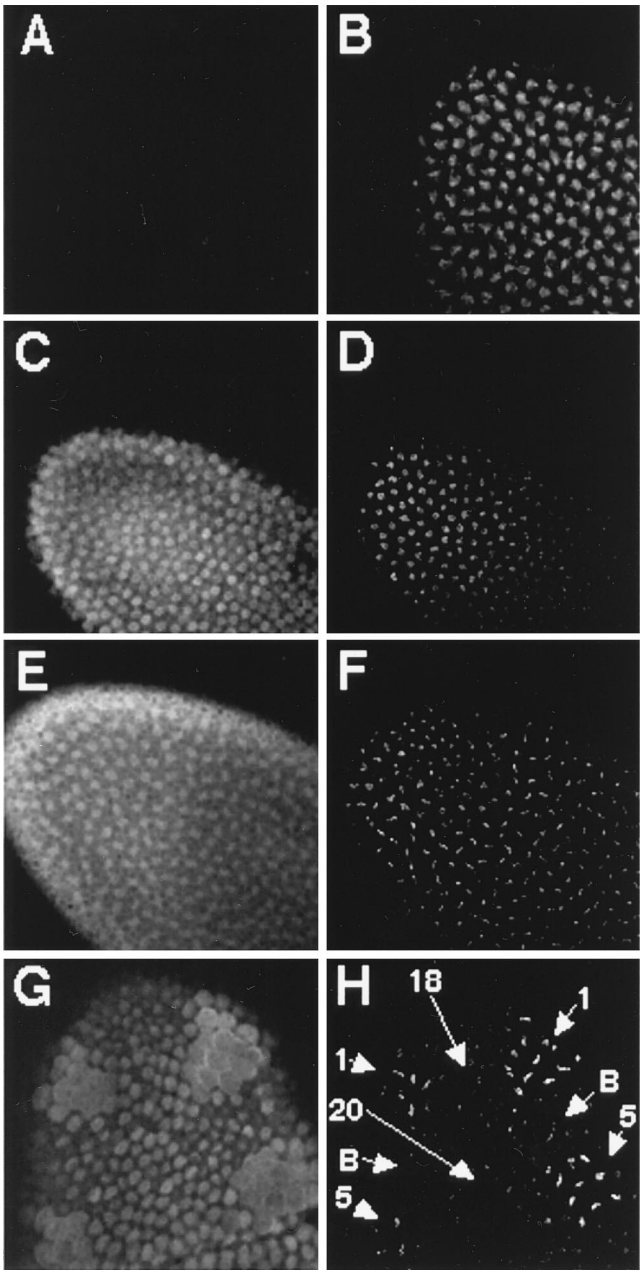

FIG. 4. Localization of p55 in *Drosophila* embryos. Embryos were subjected to indirect immunofluorescence analysis with either affinity-purified anti-p55 antibody (C, E, and G) or the corresponding preimmune serum (A), while DNA staining of the same embryos was carried out with YO-YO (Molecular Probes, Inc., Eugene, Oreg.). (A) Control staining with preimmune serum of an embryo in S phase of nuclear cycle 13; (C) p55 staining of an embryo in S phase of nuclear cycle 13; (E) p55 staining of an embryo in M phase of nuclear cycle 13; (G) p55 staining of an embryo at approximately 75 min after the start of interphase 14; (B, D, F, and H) DNA staining of the embryos that correspond to those shown in panels A, C, E, and G, respectively. The mitotic domains (9) in the embryo shown in panels G and H are indicated in panel H. The cells in mitotic domains 1 and 5 are in M phase, while the cells in domains 18, 20, and B are in G_2 phase.

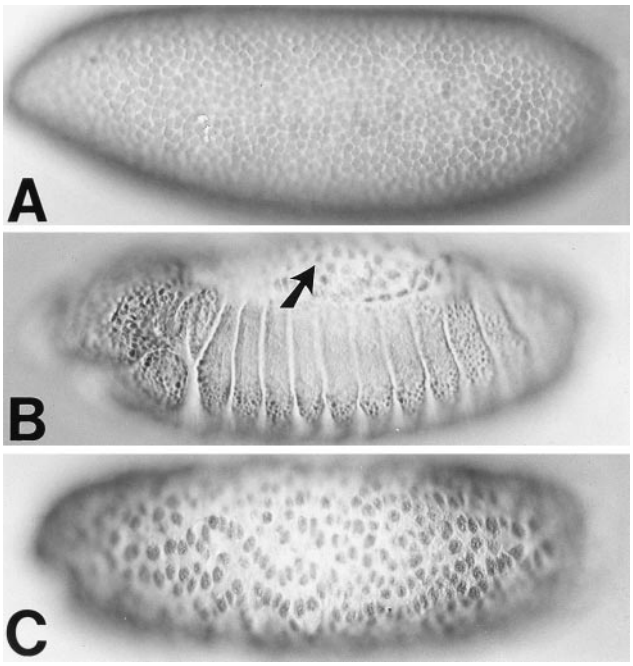

FIG. 5. Localization of dCAF-1 p55 throughout embryogenesis. Whole-mount preparations of *Drosophila* embryos were stained with affinity-purified antibodies against the p55 protein. (A) Nuclear cycle 13 (stage 4) wild-type embryo; (B) stage 14 germ band retracted wild-type embryo; (C) ventral view of a stage 14 germ band retracted mutant *string* embryo (8). The arrow in panel B indicates large cells of the amnioserosa.

6 (Fig. 7A, middle panel) to fractions 7 and 8 (Fig. 7B, middle panel). In the absence of exogenously added dCAF-1, the reaction mixture did not contain any species that are recognized by the anti-p55 antibodies (Fig. 7A, lower panel). Then, in the presence of dCAF-1 under conditions wherein DNA replication did not occur because of the omission of SV40 large T antigen from the reaction medium (Fig. 7C, middle panel), the majority of the p55 was at the top of the gradient (Fig. 7C, lower panel, fractions 2 to 4), which most likely reflects the sedimentation of native dCAF-1. However, when replication reactions were performed in the presence of dCAF-1, the majority of p55 was at the top of the gradient, while a distinct fraction of the total p55 protein reproducibly cosedimented with the newly replicated and assembled chromatin (Fig. 7B, lower panel, fractions 7 and 8). These results therefore suggest that p55 (and possibly the other dCAF-1 subunits) is associated with the newly replicated chromatin.

Histone deacetylase activity coimmunoprecipitates with p55. Lastly, because of the homology between *Drosophila* p55 and the human histone deacetylase-bound RbAp48 protein (44), we examined whether p55 is associated with a histone deacetylase activity in a crude nuclear extract from *Drosophila* embryos. As shown in Fig. 8, histone deacetylase activity specifically coimmunoprecipitated with affinity-purified antibodies against p55, but not with the corresponding preimmune serum or with a control antiserum, when histone deacetylase activity was measured by the release of [^{3}H]acetate from [^{3}H]acetyllysine histones (4). The histone deacetylase activity in the anti-p55 pellet corresponds to approximately 10% of the total histone deacetylase activity in the comparable amount of crude nuclear extract. This yield may be due to partial loss of enzyme activity during immunoprecipitation and recovery of the sample as well as the possibility that only a fraction of the histone deacetylases in the nuclear extract are associated with p55. The control immunoprecipitates did not possess significant histone deacetylase activity, as the amounts of free [^{3}H]acetate were at the background levels. Thus, consistent with the homology between *Drosophila* p55 and human RbAp48, it appears that at

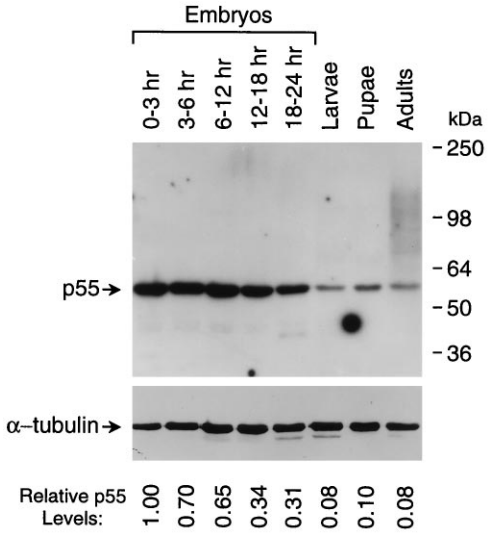

FIG. 6. dCAF-1 p55 is present throughout *Drosophila* development. Western blot analysis was performed as follows. Total protein (50 μg per lane) from *Drosophila* embryos (collected from 0 to 3, 3 to 6, 6 to 12, 12 to 18, and 18 to 24 hours after egg deposition), larvae (a mixture of first-, second-, and third-instar larvae), pupae, or adults (with approximately equivalent representation of male- and female-derived proteins) was subjected to SDS–7.5% polyacrylamide gel electrophoresis and Western blot analysis. The main panel shows the autoradiogram of the Western blot obtained with anti-p55 antibodies followed by detection with ^{125}I-labeled protein A. The lower panel shows an autoradiogram of the same blot after it was reprobed with anti-α-tubulin antibodies and detected by enhanced chemiluminescence. The data were quantitated by using a PhosphorImager and a fluorimeter (Molecular Dynamics), and the values represent the relative amount of p55, normalized to the amount of α-tubulin (which correlated with the total protein), that was present at each developmental stage.

least a fraction of the p55 in a crude nuclear extract is associated with a histone deacetylase.

DISCUSSION

To study the mechanism of chromatin assembly, we have isolated the cDNA encoding the p55 subunit of dCAF-1 and studied the properties of the protein in the context of the DNA replication-coupled assembly reaction. p55 contains seven WD repeat motifs and is homologous to two related mammalian proteins termed RbAp46 and RbAp48, the latter of which is also associated with the HD1 histone deacetylase (44). Hence, although p55 is an integral subunit of dCAF-1, it also appears to be associated with other apparently distinct proteins, including the HD1 histone deacetylase, which similarly functions with histones. The data collectively suggest that p55 functions as an important component in the linkage between DNA replication, chromatin assembly, and histone acetylation.

p55 and CAF-1. On the basis of copurification of p55 with dCAF-1 (Fig. 2) (16), the immunoaffinity purification of dCAF-1 that is active for chromatin assembly with affinity-purified antibodies against p55 (Fig. 3B and 3C), and the specific depletion of dCAF-1 activity with antibodies against p55 (Fig. 3C), we conclude that p55 is a subunit of dCAF-1. Moreover, when dCAF-1 was purified either by conventional methods (16) or by immunoaffinity techniques (Fig. 3), we observed the identical p55, p75, p105, and p180 subunits. These results therefore provide further confirmation of the assignment of the four subunits that are thought to constitute dCAF-1.

Because of the existence of the two related RbAp46 and RbAp48 proteins in mammals, we carried out numerous experiments (Southern, Northern, and Western blot analyses along with low-stringency screening of cDNA libraries) to investigate whether there might be any other p55-related genes or proteins in *D. melanogaster*, but we did not find any evidence for such genes or proteins (data not shown). The apparent absence of other p55-related genes in *D. melanogaster* suggests the divergence of *D. melanogaster* at a point earlier in evolution than the duplication event that gave rise to two homologous p55-like proteins in the higher eukaryotic species.

hCAF-1 comprises three subunits with apparent molecular masses of 150, 60, and 50 kDa (38). The isolation of cDNAs that encode hCAF-1 p150 and p60 has been described (20). hCAF-1 p60 contains seven WD repeat motifs, as does dCAF-1 p55, but the amino acid sequence of p60 is otherwise unrelated to that of p55. The p50 subunit (currently designated the p48 subunit) of hCAF-1 has been cloned, and hCAF-1 p48 was found to be identical to the human RbAp48 protein (46). Thus, dCAF-1 p55 is homologous to hCAF-1 p48. Though the specific relationship between the p180, p105, and p75 subunits of dCAF-1 and the p150 and p60 subunits of hCAF-1 remains to be determined, the properties of dCAF-1 and hCAF-1 have been compared directly and observed to be similar (16), and it thus seems likely that the human and *Drosophila* factors are related in structure and composition.

What is the function of the WD repeats in dCAF-1 p55? There are about 50 known WD repeat-containing proteins that are involved in a broad range of biological processes (28, 29), and thus the function of WD repeat proteins is not specific for histones or chromatin. The β-propeller structure of the G-protein β subunits (25, 41, 48) seems likely to be shared by other WD repeat proteins, including dCAF-1 p55. It has been postulated that the β-propeller may serve as a relatively stable structure onto which other proteins can bind. For instance, examination of the G-protein heterotrimer reveals two different sites of interaction between G_α and the β-propeller of $G_{\beta\gamma}$ (25, 48). By analogy, it is possible that p55 functions as a scaffold upon which proteins, such as other subunits of dCAF-1 or HD1, might assemble.

In our analysis of the subcellular localization of p55, we found that p55 is a predominantly nuclear protein that is present throughout *Drosophila* development (Fig. 4 to 6). In HeLa cells, it appears that the levels of the p150 and p60 subunits of hCAF-1 vary with the phase of the cell cycle and are highest during S phase, when the proteins are in the nucleus at the specific sites that are often referred to as replication foci (23). The localization of the majority of p55 may not, however, reflect the localization of the p55 that is an integral component of dCAF-1 because the p55 that is associated with dCAF-1 is likely to be a fraction of the total p55 protein in the cell. In the future, it will be interesting to compare the distribution of p55 with that of the other subunits of dCAF-1 as well as other p55-associated proteins.

Does dCAF-1 p55 interact with Rb? p55 is homologous to two mammalian proteins termed RbAp46 and RbAp48, which were initially purified and cloned on the basis of their ability to bind to a fragment of Rb (32, 33). We therefore examined whether p55 interacts with the *Drosophila* Rb-related protein, RBF (6). Specifically, we tested the ability of antibodies against RBF (which were generously provided by W. Du and N. Dyson, Massachusetts General Hospital Cancer Center, Charlestown) to coimmunoprecipitate p55 from a whole embryo protein extract, and vice versa. In these experiments, we found that affinity-purified anti-p55 antibodies apparently coimmunoprecipitated RBF, whereas the anti-RBF antibodies failed to coimmunoprecipitate p55. However, further analyses revealed that the affinity-purified anti-p55 antibodies could

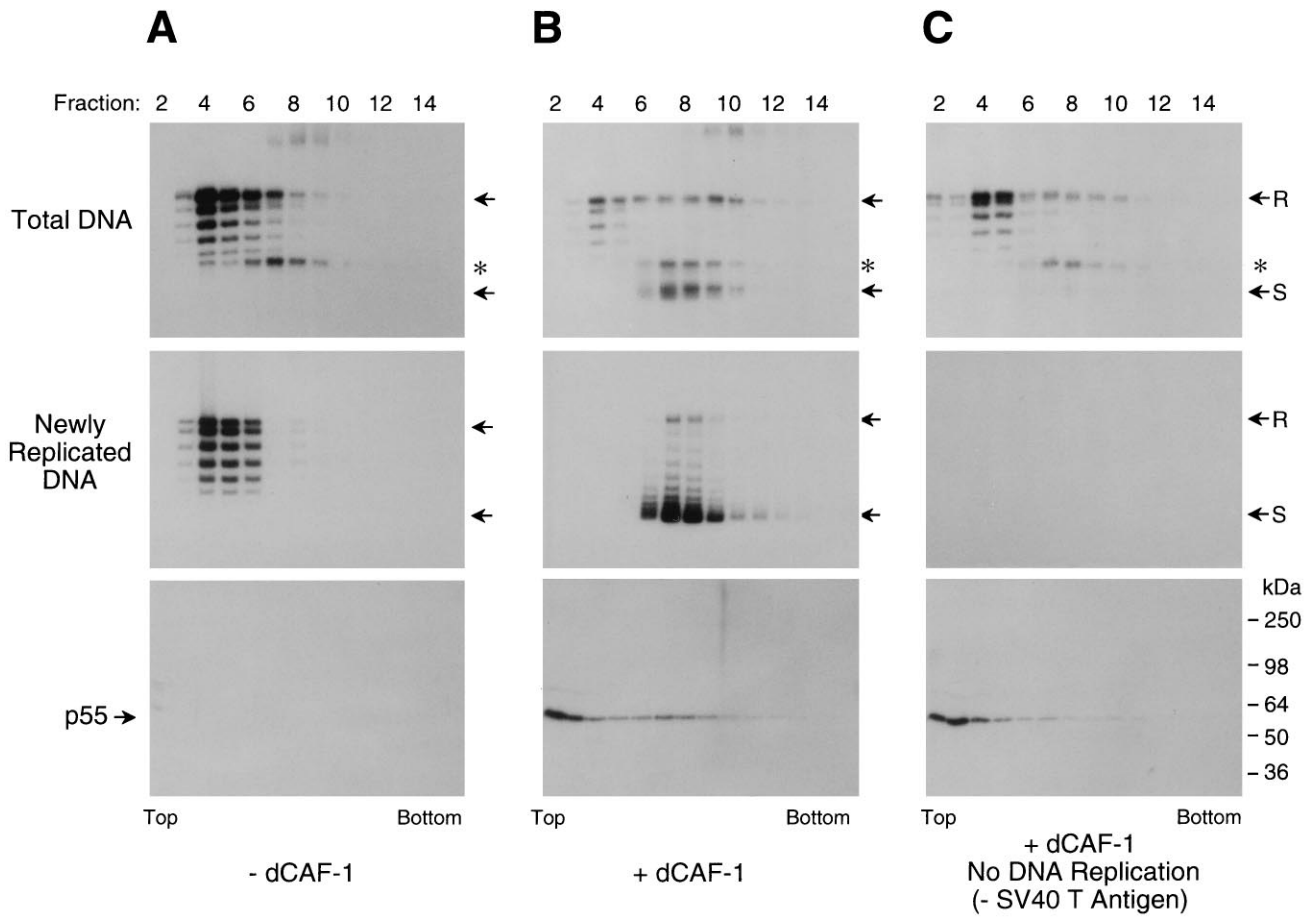

FIG. 7. Sucrose gradient sedimentation analysis of chromatin. SV40 DNA replication-chromatin assembly reactions were performed as indicated and subjected to sucrose gradient centrifugation. (A) Reactions were performed in the absence of exogenously added dCAF-1. (B) Reactions were carried out with dCAF-1. (C) Reactions were performed with dCAF-1 as for panel B except that SV40 large T antigen, which is required for DNA replication, was omitted. The upper panels are autoradiograms of Southern blots of the total plasmid DNA species after sucrose gradient sedimentation and agarose gel electrophoresis (the blots were probed with ^{32}P-labeled DNA that is complementary to the plasmid DNA used in the replication reactions). The middle panels are autoradiograms of the corresponding DNA replication products. The positions of relaxed (R) and supercoiled (S) DNA species are denoted. The lower panels are autoradiograms of anti-p55 Western blot analyses of the same sucrose gradient fractions shown in the corresponding panels above. The presence of p55 protein was detected by using ^{125}I-labeled protein A. To minimize the contribution of the radiolabeling of the newly replicated DNA in the Southern blot analysis of the total DNA, the Southern blot was performed at approximately 10 weeks (about five half-lives for ^{32}P) after the DNA replication reactions were carried out. In addition, in the Southern blots, the band indicated by an asterisk, which does not correspond to any of the replicated DNA forms (see middle panels), is observed in the absence of DNA replication and chromatin assembly and may be linear (form III) DNA that was generated by nucleases in the replication extract or an unrelated nucleic acid species that cross-hybridizes with the radiolabelled probe.

directly immunoprecipitate recombinant bacterially synthesized RBF in the absence of p55 protein (data not shown), and thus there appears to be a common epitope that is shared by p55 and RBF. The conclusion from these and other related experiments is that we were not able to detect an interaction between *Drosophila* p55 and RBF.

CAF-1 and histone acetylation. The analysis of histone modification and DNA synthesis has led to a model for histone acetylation (particularly with histones H3 and H4) and chromatin assembly wherein newly synthesized histones are acetylated, assembled into chromatin onto newly synthesized DNA, and then deacetylated (1, 15, 30, 35, 39, 40). With newly synthesized histone H4, the pattern of acetylation of lysine residues in the N-terminal tail is conserved among *Tetrahymena thermophila*, *D. melanogaster*, and humans (40), which implies a significant function for this modification of histones. It has also been found that hCAF-1 binds to newly synthesized H3 and acetylated H4 (20, 37). These and other data have led to the conclusion that CAF-1 is a core histone chaperone that delivers newly synthesized and acetylated histones to other components of the chromatin assembly machinery at the replication fork. In addition, the observation that the p55 subunit of dCAF-1 specifically associates with a histone deacetylase (Fig. 8) and is thus likely to be functionally homologous to the mammalian HD1 histone deacetylase-bound protein, RbAp48 (44), further suggests that p55 may be involved in both the assembly of the newly synthesized and acetylated histones and the deacetylation of the histones in the newly assembled chromatin. It is possible, for instance, that there exists, perhaps even transiently, a complex containing both CAF-1 and HD1. Moreover, it should be noted that human HD1 is similar to yeast Rpd3p, which has been found to affect the transcriptional regulation of genes in *S. cerevisiae* (47). Hence, p55 appears to be involved at the interface of DNA replication, chromatin assembly, histone modification, and transcriptional regulation.

p55 is associated with newly assembled chromatin. By sucrose gradient sedimentation analysis, we have found that a fraction of the total p55 protein is associated with chromatin that is generated during dCAF-1-mediated, DNA replication-coupled chromatin assembly (Fig. 7). This association of

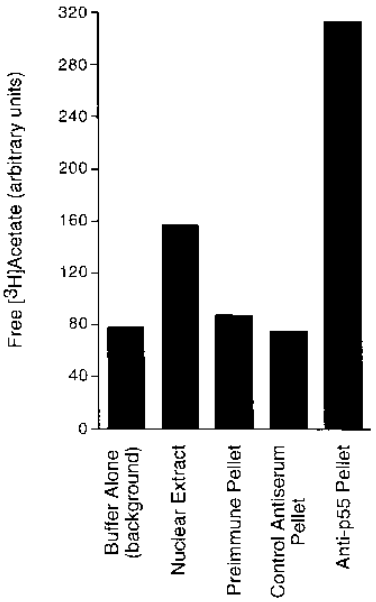

FIG. 8. Coimmunoprecipitation of histone deacetylase activity with p55. A crude nuclear extract derived from *Drosophila* embryos was subjected to immunoprecipitation with antibodies against p55, the corresponding preimmune serum, or an unrelated (control) antiserum, and the resulting pellets (comprising antibodies and associated proteins bound to protein A-Sepharose; each derived from 165 μl of nuclear extract) were tested for histone deacetylase activity, as monitored by the release of [^{3}H]acetate from [^{3}H]acetyllysine histones (4). The amount of free [^{3}H]acetate that was present upon incubation with buffer alone was measured to determine the background. The total histone deacetylase activity in the crude nuclear extract (5 μl) is also shown.

dCAF-1 p55 with newly assembled chromatin in vitro is consistent with the observation that a CAF-1-like activity is a component of the nonhistone proteins that copurify with SV40 minichromosomes isolated from nuclei of virus-infected cells (24). Hence, these experiments independently suggest that CAF-1 has an affinity for chromatin. However, the specificity of the interaction and the functional consequences of CAF-1 binding remain to be clarified. It might be postulated, for instance, that p55 or dCAF-1 is incorporated into chromatin during DNA replication and then subsequently acts to recruit histone deacetylases to the newly assembled chromatin. As the remainder of the factors involved in chromatin assembly and modification are purified and cloned, it will become possible to clarify these and other questions to provide key insight into a centrally important biological process.

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