**Drosophila** nucleosomes contain an unusual histone-like protein

(Triton DF-16 gel electrophoresis/tryptic peptide maps)

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**ABSTRACT** Mononucleosomes prepared from *Drosophila melanogaster* nuclei contain the four core histones H2A, H2B, H3, and H4 plus an additional histone-like, acid-soluble, chromosomal protein. It is probably the protein designated D2 by Alfageme *et al.* (Alfageme, C. R., Zweidler, A., Mahowald, A. & Cohen, L. H. (1974) J. Biol. Chem. 249, 3729–3736). D2 elutes with histone H2A from a Bio-Gel P-100 column, but can be distinguished electrophoretically from H2A and from the other standard *Drosophila* core histones. The amino acid composition of D2 resembles the compositions of H2A and H2B. However, peptide mapping reveals that D2 is not a simple sequence variant of either H2A or H2B. D2 is present in nuclei from embryos and adult heads, and thus is not restricted to a narrowly defined developmental period. It is present in *D. melanogaster* and *D. virilis* and thus appears to be conserved during the evolution of *Drosophila*. D2 is present in *D. melanogaster* chromatin with an approximate frequency of one molecule per five nucleosomes, and must therefore be associated with a subset of nucleosomes. The function of this protein is not known. Its presence in nucleosomes, evolutionary conservation, and comparatively large abundance all suggest that it is an important nucleosomal element. It will be interesting to learn whether this histone-like protein is encoded in a subset of the *Drosophila* histone gene cluster or is encoded separately.

Histones are major structural components of the basic repeating subunit of chromatin, the nucleosome (1). Core histone sequences are remarkably conserved during evolution. Nonetheless, a number of histone variants are known (2–4). Synthesis of core histone variants is stage-specific during sea urchin development (3, 4), tissue-specific in mammals (5), and correlated with erythroid differentiation in Friend leukemia cells (6, 7). These observations suggest that nucleosome heterogeneity is related to the functional specialization of specific elements of chromatin. In this context, it may be important that mononucleosomal isolates from *Drosophila* chromatin contain large amounts of a histone-like protein that is not a standard core histone. This protein was first identified by Alfageme *et al.* (8), and designated D2, or "*Drosophila* 2."

In this communication, we demonstrate that D2 is nucleosomal and histone-like, yet is readily distinguished from each of the four "standard" core histones. It is also conserved during the evolution of *Drosophila*. The curious features of D2 raise questions about histone evolution and the organization of histone genes in *Drosophila*.

**MATERIALS AND METHODS**

**Purification of Nuclei.** *D. melanogaster* embryo nuclei and adult head nuclei were prepared with three modifications of published procedures (9, 10): (i) buffer A of Hewish and Burgoyne (11), containing 1 mM EDTA, 0.2 mM ethylene glycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), and 0.2 mM phenylmethylsulfonyl fluoride was used for the nuclear isolation; (ii) the crude nuclear pellet was washed in buffer containing 0.1% Triton X-100; (iii) the detergent-washed nuclei were then resuspended and recentrifuged three times in buffer lacking Triton X-100.

**Purification of Mononucleosomes.** Nuclei were suspended in buffer A of Hewish and Burgoyne (11), made 4 mM in CaCl₂, and digested at 25°C to 25–29% acid solubility with staphylococcal nuclease (EC 3.1.31.1). Digested nuclei were lysed by Noll and Kornberg (12). Digests were fractionated by sedimentation through linear (5–20%) sucrose gradients containing 0.5 M NaCl (13) or by electrophoresis in 5% acrylamide gels (14).

**Analysis of Nucleosomal DNA.** DNA was extracted from pooled, sucrose-gradient-purified nucleosomes (15), omitting RNase treatment. DNA was released from electrophoretically purified nucleosomes with 1% NaDodSO₄ and proteinase K at 100 μg/ml. DNA was electrophoresed on 5% acrylamide slab gels (16) and visualized by staining with ethidium bromide.

**Analysis of Nucleosomal Proteins.** Gradient-purified nucleosomes were precipitated with 10 mM MgCl₂ (17) and extracted with protamine/urea/acetic acid (18). The released chromosomal proteins were applied directly to acetic acid/urea/Triton DF-16 gels (see below). Electrophoretically purified nucleosomes were electrophoresed into dialysis bags and extracted with 0.25 M HCl (8). The extracted proteins were analyzed by two-dimensional electrophoresis (see below).

**Purification of Histones.** *D. melanogaster* embryo histones were extracted from nuclei (10), chromatographed on Bio-Gel P-100, and assayed by absorbance at 230 nm (19). Histones in the A₂₅₀ peaks were precipitated with trichloroacetic acid (8). The H2A peak (ca. 5 mg) was then fragmented by electrophoresis at 140 V on a cylindrical gel (1.0 × 20 cm), containing 12% acrylamide, 0.37% Triton X-100, 6.5 M urea, and 5% (vol/vol) acetic acid (8). Eluted protein was collected in 2-ml fractions and assayed (20).

**Electrophoresis of Histones.** Histones were separated electrophoretically on acid/urea gels containing 12% acrylamide and 6 M urea (21), acid/urea gels containing 0.5% Triton DF-16 (8), or 18-cm NaDodSO₄ gels (22). For two-dimensional electrophoresis, acid/urea or acid/urea/Triton gels were the first dimension, while NaDodSO₄ gels were the second dimension. Gels were stained with Coomassie blue. For quantitation of the relative amounts of specific histone fractions, gels were stained with Procion blue (23) and scanned densitometrically.

**Amino Acid Analysis and Tryptic Peptide Mapping.** Protein hydrolysis and amino acid analysis were performed according to Moore and Stein (24), except that 80 mM 2-mercaptoethanol was included in the hydrolysis (25). Samples were hydrolyzed for 24 and 48 hr. Values for serine and threonine were corrected for loss during hydrolysis. Tryptophan, cysteine, cystine, and amide were not determined. Tryptic maps of histones were prepared on microcrystalline cellulose plates and visualized with fluorescamine (7).
RESULTS

*D. melanogaster* histones eluted from a Bio-Gel P-100 column in the order H1, H2A, H2B, H3, and H4 (26). Electrophoresis revealed that the H2A peak contained H2A plus an additional component. This component, which made up about 10% of the H2A peak, migrated with H3 in acid/urea gels (Fig. 1A). In Triton/acid/urea gels, it migrated as a tightly spaced doublet between H2A and H3 (Fig. 1B). The two subfractions com-
the most ranged composition and H2A, H3 and homology simple sequence not present D2 mixtures show that maps percentage lacks methionine basic of (Table 1) resembles the good 295% H2B NaDodSO4 gel electrophoresis (Fig. 1D), histones by Triton/acid/urea gel electrophoresis (Fig. 1B), (26). It this basis of reflect dently both from together in grated in NaDodSO4 gels (Fig. 1C). Because they eluted to- gether from a Bio-Gel column and migrated as a single band in both acid/urea and NaDodSO4 gels, these subfractions evidently reflect microheterogeneity within a single protein. On the basis of its mobilities in acid/urea and Triton/acid/urea gels, this protein is tentatively identified as the chromosomal protein D2 (8). D2 has a mass of approximately 13,400 daltons (26). It can be distinguished from the standard Drosophila histones by Triton/acid/urea gel electrophoresis (Fig. 1B), NaDodSO4 gel electrophoresis (Fig. 1D), or two-dimensional gel electrophoresis (Fig. 1E).

NaDodSO4 gel electrophoresis revealed that the H2A and H2B samples used for amino acid analysis and tryptic mapping were ≥95% pure, whereas the D2 samples were ≥90% pure. D2 was slightly contaminated with oxidized H2A (Fig. 2F).

The amino acid compositions obtained for H2A and H2B are in good agreement with previously published values (8). D2 resembles the slightly lysine-rich H2A and H2B histones in ratio of basic to acidic residues and ratio of lysine to arginine residues (Table 1) but differs from both these proteins in mole percentage of several amino acids. In contrast to H2A and H2B, D2 lacks methionine and contains 3.6 mol % histidine. Tryptic maps of H2A, H2B, D2, H2A/D2 mixtures, and H2B/D2 mixtures show that a majority of the peptides derived from D2 are not present in H2A or H2B (Fig. 2). Therefore, D2 is not a simple sequence variant of either H2A or H2B. Because some D2 peptides comigrate with H2A peptides, partial sequence homology between D2 and H2A is possible. The amino acid composition and tryptic maps of D2 do not resemble those of H3 and H4 (8, 26). Thus D2 is not a simple sequence variant of H2A, H2B, H3, or H4.

DNA from sucrose-gradient-purified D. melanogaster mononucleosomes ranged in size from 63 to 163 base pairs. The most prominent fragments were 146 and 163 base pairs. Fragments smaller than 146 base pairs accounted for approximately 30% of the DNA (Fig. 3A). Nonhistone chromosomal proteins and H1 were depleted in the mononucleosomes. In contrast, the relative amounts of D2 and the four standard core histones H2A, H2B, H3, and H4 were similar in mononucleosomes and untreated nuclei (Fig. 3B).

Comparable results were noted with electrophoretically purified mononucleosomes. DNA from the most rapidly migrating mononucleosomal component of chromatin digests (component I, Fig. 4A) was ≤163 base pairs (data not shown). Two-dimensional gel electrophoresis revealed that component I contained undiminished amounts of D2 (Fig. 4 B and C). Because mononucleosomes prepared in either of two ways and nuclei contain similar proportions of D2, D2 is a nucleosomal protein.

Proteins with chromatographic and electrophoretic prop- erties indistinguishable from those of D. melanogaster D2 were

![Fig. 3. Gel electrophoresis. (A) DNA extracted from sucrose-gradient-purified D. melanogaster embryo mononucleosomes. Electrophoresis was from left to right in a 5% acrylamide gel. A den- sitometric tracing of a photographic negative of the gel is shown above the gel. The number of base pairs (given above each peak) in the bands was measured relative to phage 4X174 restriction endonuclease fragments (26). (B) The protamine/urea/acetic acid-extractable proteins from untreated nuclei and sucrose-gradient-purified mononucleosomes. Electrophoresis was from left to right in 20-cm-long acid/urea/Triton DF-16 gels.](image-url)
FIG. 4. (A) Densitometric tracing of a 5% polyacrylamide gel containing a 25% staphylococcal nuclease digest of D. melanogaster embryo nuclei. The gel was stained with Coomassie brilliant blue R. The hatched area indicates the region chosen for further analysis. The corresponding region in preparative gels was located by staining with ethidium bromide. (B) Two-dimensional gel electrophoresis of histones extracted from intact D. melanogaster embryo nuclei. The first dimension was acid/urea gel electrophoresis in an 18-cm gel. The second dimension was NaDodSO4 gel electrophoresis. H1 is outside the region of the gel shown. (C) Two-dimensional gel electrophoresis of the histones extracted from electrophoretically purified D. melanogaster mononucleosomes (corresponding to the hatched area in A). Conditions of electrophoresis were as in B. H1 was not present in these mononucleosomes.

present in acid extracts of nuclei from D. virilis (Fig. 5A). Tryptic peptide maps confirmed the identity of the D. virilis proteins (26). Therefore, D2 has been conserved during the speciation of Drosophila. D2 is present in similar amounts in embryos and adult heads (Fig. 5B; ref. 26). It seems likely, therefore, that D2 functions throughout the life of Drosophila.

DISCUSSION

Similar amounts of D2 are found in nuclei and mononucleosomes purified from nuclei by two alternative procedures. D2, therefore, is a component of nucleosomes. Unlike the high mobility group (HMG) proteins (27, 28), or the acidic–basic Drosophila chromosomal protein D1 (29), D2 is associated with nucleosomes prepared in 0.5 M NaCl. The amino acid composition of D2 also distinguishes it from these proteins. D2 resembles the slightly lysine-rich histones H2A and H2B in ratio of basic to acidic amino acid residues and ratio of lysine to arginine residues. It can, however, be distinguished from both H2A and H2B by electrophoresis, amino acid analysis, and tryptic peptide mapping. D2, although histone-like, is not a simple sequence variant of a standard Drosophila histone. Rat liver chromatin contains the unusual chromosomal protein, A24, which features an isopeptide linkage between histone H2A and ubiquitin (30, 31). Because D2 and H2A have limited sequence homology, and because A24 is much larger than D2, it does not appear likely that D2 and A24 are analogous. Because D2 shows little homology with H2B, H3, and H4, and because D2 is similar in apparent molecular weight to these proteins, it also appears that D2 cannot be an A24-like complex of nonhistone chromosomal protein and any standard core histone. The source of the microheterogeneity in D2 revealed by acid/urea/Triton DF-16/polyacrylamide gel electrophoresis is unknown. It may be the result of posttranslational modification, or, alternatively, sequence heterogeneity similar to that observed in mammalian or chicken histones (2, 32).

It is not known whether D2 is present in nucleosomes in addition to a full complement of the four core histones or, alternatively, substitutes for a core histone. The similarities of size and amino acid composition between D2 and the slightly lysine-rich histones suggest that D2 might substitute for either H2A or H2B. In this respect, D2 may be analogous to the vertebrate proteins M1 and M2 (5, 32). It has been suggested that these histone-like proteins substitute for H2A (14, 32). Interestingly, their amino acid compositions closely resemble the composition of D2.

Like the nucleosomal core histones, D2 is conserved in evolution. The tryptic peptide maps, chromatographic properties, and electrophoretic properties of D2 from the distantly related species D. melanogaster and D. virilis are similar and may be
identical. Because D2 closely resembles the mammalian proteins M1 and M2, it is possible that proteins homologous to D2 are important determinants of chromatin structure in many eukaryotes.

Stage-specific switches in synthesis of histone primary sequence variants occur during sea urchin embryogenesis (3, 4). Because D2 is a major chromosomal protein in embryos and in adult heads, it probably is not a comparable stage-specific protein. Diploid nuclei from D. melanogaster contain ca. 2 X 10^6 molecules of D2. These large quantities are, however, insufficient for D2 to be present once per nucleosome. We calculate that D2 is present in D. melanogaster with an approximate frequency of one molecule per five nucleosomes. Therefore, D2 must be associated with a subset of nucleosomes. It is not yet known whether D2 is distributed uniformly throughout chromatin or restricted to particular regions of chromatin. Anti-D2 antibodies will be useful in such an analysis.

The Drosophila histone genes are encoded in a tandem array that is located at 2:39D-E on the polytene salivary gland map (33). It will be interesting to learn whether D2 is encoded in a subset of the histone gene clusters or in encoded separately. It will also be interesting to learn whether the syntheses of D2 and the standard histones are coordinated.

D. melanogaster mononucleosomes contain a similar D2/core histone ratio to untreated nuclei. Although a majority of the DNA isolated from the monosomes was ≤1.5% base pairs, the monosomes contained some DNA of sufficient size to retain all the histones (12). These data, therefore, demonstrate that D2 is nucleosomal. They strongly suggest, but do not conclusively prove, that D2 is associated with the nucleosomal core particle rather than the linker DNA between nucleosomes (1).

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