Histone Molar Ratios among Different Electrophoretic Forms of Mono- and Dinucleosomes*

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The relative molar ratios of each of the histone classes and protein A24 have been determined in nuclei, chromatin, and different electrophoretic forms of mono- and dinucleosomes of cultured mouse cells. For this purpose, [3H]lysine- and [14C]arginine-labeled cells were used for sample preparations, and stoichiometries were estimated from protein radioactivity profiles and known amino acid compositions following sodium dodecyl sulfate (SDS)-gel electrophoresis. The results demonstrate that variable upper limits of one and two histone H1 molecules exist per mono- and dinucleosome, respectively. However, isolated nuclei contain less than one copy of histone H1 per nucleosome. In addition, among the chromatin subfractions studied, histones H3, H2B, and H4 are essentially equimolar, while histone H2A is less than equimolar by 19 ± 9%. This latter finding offers direct support to the proposal of Goldknopf et al. (25) that protein A24 replaces histone H2A in the octamer protein core of the nucleosome, since about 10% of the total histone H2A of cultured mouse cells is in the form of protein A24 and is present in nucleosomes. From the results of the present study, it is concluded that electrophoretic fractionation of mono- and dinucleosomes is not due to variable molar ratios or amounts of the four smaller histone classes, but depends in part on DNA length, the number of associated histone H1 molecules, and non-histone chromosomal proteins.

There has been a large body of evidence supporting the original proposals of Kornberg (1) and Van Holde et al. (2) that an octamer composed of two copies each of histones H2A, H2B, H3, and H4 serves as the fundamental structural unit of chromatin (3–17). However, a number of findings have been published which suggest the possibility that heterogeneity may exist in the histone composition of nucleosomes of mammalian chromatin. These include: (i) the observation that histones H3 and H4 alone can reconstitute many characteristics of chromatin structure, including a portion of the x-ray diffraction pattern (18, 19), arrays of nucleoprotein particles as visualized by electron microscopy (20–22), superhelical density (20, 22, 23), and protection of certain specific lengths of DNA against nuclease digestion (10, 11, 19); (ii) the findings that nucleosomes of rat liver which have been spread on electron microscope grids exhibit differential and limited susceptibility toward reaction with antisera prepared against specific histones (24); (iii) the discovery of a novel protein, termed protein A24, which is present in nucleosomes and consists of histone H2A coupled covalently by an isopeptide linkage at lysine 119 to a nonhistone protein termed ubiquitin of molecular weight 8451 (see Refs. 25 and 26), and finally the subject of this investigation, (iv) the observation that the nucleoprotein products resulting from partial digestion of chromatin with micrococcal nuclease can be resolved electrophoretically into distinct classes of mono- and dinucleosomes (12, 27–31).

The present study was undertaken to address the question of possible heterogeneity in the amounts of the four smaller histones present in electrophoretically fractionated mono- and dinucleosomes of cultured mouse cells. Of further interest was to determine the previously unestablished stoichiometries of histone H1 among different electrophoretic forms of nucleoprotein species. In addition, we have investigated the content and distribution of protein A24, the component mentioned above which contains within its structure histone H2A. Goldknopf et al. (25) have suggested that this unique branched protein composed of both a histone and nonhistone protein may replace histone H2A in the octamer protein core of the nucleosome and, thereby, create a subset of nucleosomes which may have important biological roles in chromatin fiber packaging. Commensurate with this view is the knowledge that the level of nucleolar protein A24 is inversely proportional to ribosomal gene activity (32, 33), suggesting the possibility that protein A24 may serve a role in chromatin condensation and hence genetic inactivation.

In this report, we demonstrate that an upper limit of one histone H1 molecule exists per histone octamer, but that isolated nuclei contain less than one copy of histone H1 per nucleosome. We show further that the nucleosomal core histones are predominantly equimolar among electrophoretically fractionated nucleoprotein species except for histone H2A, which is present in reduced amounts (0.81 ± 0.09 mol/mol of histone H4). Since about 10% of the total histone H2A of cultured mouse cells participates in protein A24 biosynthesis and is present in nucleosomes, the observed substoichiometry offers direct support to the proposal of Goldknopf et al. (25) that protein A24 replaces histone H2A in the octamer protein core of the nucleosome.

EXPERIMENTAL PROCEDURES

Enzymatic Preparation of Chromatin from Labeled Mouse Cells—Cultured mouse mastocytoma (line P815) cells were labeled continuously during exponential growth for approximately eight generations with either 5 μCi/ml of L-4,5-[3H]lysine and 0.17 μCi/ml of L-[guanido-14C]arginine (47 Ci/mmol and 50 mCi/mmol, respectively) or 1 μCi/ml of [methyl-3H]thymidine plus 5 μCi/ml of unlabeled thymidine and 0.17 μCi/ml of L-[guanido-14C]arginine (50 Ci/mmol and 50 mCi/mmol, respectively). Isotopes were purchased from...
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Schwarz/Mann. Cultures were maintained in suspension at a density from 0.2 to 1 x 10⁶ cells/ml as described elsewhere (30). Chromatin was prepared from isolated nuclei following digestion with micrococcal nuclease, and DNA and acid-soluble material were assayed as reported previously (30).

**Gel Electrophoresis of Chromatin**—Enzymatically prepared chromatin was separated by electrophoresis at 4°C using 5% acrylamide (20:1 acrylamide:N,N'-methylenebisacrylamide) slab gels (15 x 14 x 0.3 cm with 0.8-mm sample wells) with 6.4 mm Tris, 3.2 mM sodium acetate, 0.32 mM EDTA, pH 8.0, as the buffer system, with recirculation. Gels were subjected to pre-electrophoresis for 2 h at 250 V, and freshly prepared chromatin samples (20, 50, or 100 µg as DNA in 5% polyacrylamide, 2 mM EDTA, 0.01% bromphenol blue, pH 7.2) were separated electrophoretically for 20 min at 60 V followed by 5 h at 250 V. Gels were stained by soaking for 1/2 h in an aqueous solution of 1 µg/ml of ethidium bromide and nucleoprotein bands (0.15 to 0.3 cm) were excised using a razor blade during visualization under weak illumination by short wave ultraviolet light. Nucleoprotein components MI, MV, V, and MIII, DII, DIII, DIII, DIII, DIII (see Fig. 2) were excised from tracks loaded initially with 100- and 50-µg samples, respectively. Band locations were facilitated by flanking preparative slots with 20-µg sample loads.

**Gel Electrophoresis of DNA and Protein**—The homogeneity of nucleoprotein components which had been excised from 5% acrylamide gels was assayed by performing DNA electrophoresis as follows. Gels which were 0.8 x 0.3 x 0.15 cm were soaked for 1 h at 37°C in 10 mM Tris, 30 mM sodium phosphate, 10 mM EDTA, pH 7.8, containing 1% SDS and 100 µg/ml of pronase. Slices then were fit into sample wells of 6% acrylamide, 0.1% SDS slab gels and subjected to electrophoresis with buffer recirculation as detailed elsewhere (29).

The histones of various nucleoprotein complexes were separated by 18% acrylamide, 0.1% SDS slab gels (27 x 15 x 0.3 cm) as described by Thomas and Kornberg (6). From two to three gel slices of a given nucleoprotein species were positioned together in 0.8-cm wells of preformed 6% acrylamide stacking gels. Samples were placed in alternate wells and were flanked with 60-µg loads of cytochrome c. Gel slices were surrounded with a solution heated to 100°C containing 8 M urea, 0.125 M Tris, 5% 2-mercaptoethanol, 1% SDS, pH 6.8. After 1 h at room temperature, the tray buffer was added gently and electrophoresis was performed at 10 watts/gel for 30 to 32 h at 8°C. Periodically during the run, cytochrome c was again loaded. This provided visual markers for cutting the tracks correctly prior to gel slicing and permitted the full lengths of gels to be used for sample separation since histone H4 and cytochrome c have the same electrophoretic mobilities. Control experiments have demonstrated that over 99.9% of the labeled protein leaves both the 5% acrylamide gel slices and the stacking gels using the above procedures.

**Gel Slicing and Scintillation Counting—**Sample tracks were sliced from 18% acrylamide, 0.1% SDS slab gels using cytochrome c bands in adjacent wells as a guide. Tracks were placed between Parafilm membranes that were separated by Tygon spacers and frozen on a block of dry ice. A Mickel gel slicer which was modified to cut 1.8-cm-wide gels was used to cut 1-mm gel slices. Samples were placed in 20-ml glass scintillation vials and counted after 48 h of soaking at room temperature in 9 ml NCS/toluene scintillation mixture (34). All protein peaks were counted to an error of ±5% and data were corrected for channel overlaps.

**Curve Analysis**—The mass proportions of Gaussian components were estimated by the use of a six-channel DuPont curve analyzer to fit normal curves to data points of radioactivity versus gel slice number. Unresolved profiles were cut, weighed, and individual peak weights were calculated from the best fit mass proportions determined by prior curve analysis.

**Amino Acid Analysis**—Isolated nuclei of cells labeled with 3H-lysine and L-[3H]arginine as described above were extracted with 0.4 N H₂SO₄, and the resulting basic proteins were precipitated with ethanol (34). The sample was hydrolyzed and fractionated on a Durrum D-500 amino acid analyzer as described elsewhere (35). As shown in Fig. 1, over 95% of the incorporated ³H and ¹⁴C radioactivities co-chromatograph with lysine and arginine, respectively. It is concluded that the radioactive amino acids supplied to cells are not contaminated significantly with other radioactive amino acids, and that during cell growth the precursors are not metabolized to other radioactive amino acids which in turn get incorporated into nuclear basic proteins.

The abbreviation used is: SDS, sodium dodecyl sulfate.

**RESULTS AND DISCUSSION**

**Experimental Approach**—In the present study, the molar ratios of the histones and protein A24 have been estimated by a radioisotopic approach. Exponential cultures of mouse cells were labeled continuously for eight generations with [³H]-lysine and [¹⁴C]arginine. Under the conditions used, over 97% of the radioactivity of each label which was incorporated into nuclear basic proteins was incorporated as the correct amino acid without redistribution (Fig. 1; see "Experimental Procedures"). Therefore, provided that the histones and protein A24 can be quantitatively recovered after separation from themselves and non-histone proteins, the relative molar ratios of these proteins in various chromatin subfractions can be estimated independently from the amount of incorporation of each isotope and the known amino acid compositions of the various protein species (36, 37). This method of stoichiometry estimation has been used in the present investigation on the assumption that different histones and protein A24 are synthesized from precursor pools of lysyl- or arginyl-tRNA molecules of similar specific activities.

It should be noted that during the course of the studies described here two reports appeared which utilized isotopic approaches for estimating the molar ratios of histones. One study concluded that chick erythroblast nuclei contain equimolar amounts of the four smaller histones (13), while the other investigation reported that equimolar ratios of the nucleosomal core histones exist among mono-, di-, tri-, and tetraneucleosomes of cultured Chinese hamster cells (15). However, due to difficulties in the separation of histone H2A from histone H2B, both of these earlier studies treated histones H2A and H2B as one component instead of two separate species. Our study differs in several respects from these earlier investigations. Since it has been suggested that protein A24 may replace histone H2A in the core of the nucleosome (25), we have tested this hypothesis by utilizing methods which permit individual determinations of the molar ratios of histone H2A, protein A24, and histone H2B. Furthermore, we have studied the molar ratios of these and other histone proteins in nucleoprotein components which have been fractionated electrophoretically, thus offering every opportunity to detect heterogeneity in protein composition should it exist. Finally, we have determined the molar ratios of histone H1 among electrophoretically fractionated mono- and dinucleosomes, thus providing the first rigorous account of the stoichiometry of lysine-rich histones among these components.

**Preparation of Nucleoprotein Species**—In order to prepare samples for stoichiometry estimates, labeled mouse chromatin

![Fig. 1. Distribution of radioactive amino acids in nuclear basic proteins after prolonged cell growth. Nuclear basic proteins were prepared from cells labeled for 8 generations with [³H]lysine and [¹⁴C]arginine. An acid hydrolysate was analyzed on a Durrum D-500 amino acid analyzer (35).](image-url)
which had been partially digested with micrococcal nuclease was separated by electrophoresis using 5% acrylamide slab gels. As shown in Fig. 2A, mononucleosomes are fractionated into two major and several minor components, which have been termed MI, MII, ... MV (see Ref. 31) as an abbreviated nomenclature to that described elsewhere (29, 30); these mononucleosomes are of interest because they originate, in part, from poly-nucleosomal arrays with different DNA repeat lengths (29–31). In agreement with previous reports (27, 28), using 5% acrylamide gels dinucleosomes are separated into three components, which will be referred to in the present study as D1, D2, and D3 (Fig. 2A). These dinucleosomes, in part, are the immediate precursors of MI, MII, and MV, respectively (31). Samples for stoichiometry estimates were excised from preparative 5% acrylamide gels in the areas indicated by the letters a to f as shown in Fig. 2A, which correspond to nucleoprotein species D1, D2, D3, MV, MIV–V, MIII, and MI. Particular care was taken to avoid cross-contamination between dinucleosomal components by excising D1 and D2 samples only from more slowly and more rapidly migrating regions of their bands, respectively (Fig. 2A, regions a and c). The results of earlier experiments which fitted densitometric scans of the dinucleosome region to Gaussian curves aided in determining the regions to be excised so as to preclude significant cross-contamination (Fig. 2A, dashed lines). Fig. 2B shows DNA fragment patterns of nucleoprotein species prepared in this manner; dinucleosomal components consist of unique but partially overlapping distributions of DNA lengths, while mononucleosomal components consist of unique and nonoverlapping DNA length distributions. These results attest to the purity of the samples used for stoichiometry estimates, since identical loads and electrophoretic conditions were used for preparation of nucleoprotein species for purposes of subsequent separation of chromosomal proteins. The average DNA fragment sizes of components a to f, estimated relative to the mobilities of Hae III endonuclease-treated bacteriophage PM2 DNA standards (38), are 367, 342, 305, 212, 172, and 150 base pairs, respectively (Fig. 2).

Resolution of Chromosomal Proteins—For purposes of stoichiometry estimates, labeled chromosomal proteins of the nucleoprotein components described above were separated by electrophoresis using 27-cm 18% acrylamide, 0.1% SDS slab gels. In order to ensure quantitative recovery of proteins, gel slices containing nucleoprotein species were placed directly in sample wells and then soaked in SDS sample buffer prior to electrophoretic separation. Control experiments demonstrated that over 99.9% of the total labeled protein migrated into separating gels using this procedure. After electrophoretic separation, individual sample tracks were excised, frozen, and sliced into 1-mm sections; fixation and staining techniques were avoided because the results of preliminary experiments suggested that differential losses of chromosomal proteins occur during these operations.

Radioactivity profiles of the histones and protein A24 for samples prepared from whole nuclei, digested chromatin, D1, D2, D3, MV–V, MIII, and MI are shown in Fig. 3. A to H, respectively. Approximately 1.6 X 10^7 data points have been omitted to facilitate ease in reproduction. The assignment of the position of protein A24 was by the following criteria. (i) Relative to protein standards, the apparent molecular weight of the putative protein A24 band is 27,000, in agreement with the published value for protein A24 as determined by SDS-gel electrophoresis (39). (ii) The estimated molar ratio of lysinearginine residues of the putative protein A24 band agrees with the value calculated from the published amino acid sequences of histone H2A and ubiquitin (36, 37), as estimated from 3H:3C radioactivity ratios of the putative protein A24 band. (iii) The putative protein A24 band is not extracted from chromatin by 0.6 M NaCl treatment, thus ruling out high mobility group non-histone proteins (40) as contributing to the assignment of this component. (iv) Earlier two-dimensional mapping experiments using acid-urea gels to display nucleosomal proteins suggested that MI contains greatly reduced amounts of protein A24 (30); as shown in Fig. 3F the band assigned as protein A24 is essentially absent from MI. (v) Finally, separation of acid-soluble chromatin proteins by acid-urea Triton X-100 gel electrophoresis (41), followed by electrophoresis in a second dimension using SDS-gels, reveals that the band assigned as protein A24 is composed of two components which migrate slower than histone H2A in the first dimension (not shown).

Thus the dramatic mobility shift observed upon Triton X-100 binding to histone H2A is observed also for protein A24, and the two variant forms of histone H2A which are found in this cell line (30) both participate in protein A24 biosynthesis.

In order to estimate the relative molar ratios of the individual histones and protein A24, all radioactivity profiles were fitted to Gaussian curves using a DuPont curve analyzer. Fig. 4 shows the pattern obtained upon curve analysis of the data.
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ELECTROPHORETIC MOBILITY

Fig. 3. Electrophoretic patterns of histones and protein A24 of various chromatin subfractions. A, nuclei; B, digested chromatin; C, D1; D, D2; E, D3; F, MIV-V; G, MIII; and H, MI. The above samples, prepared from cells labeled for 8 generations with {\[^{14}\text{C} \text{ arginine and } ^{3}\text{H} \text{ lysine}, were separated by electrophoresis using 27-cm 18% acrylamide, 0.1% SDS slab gels. Sample tracks were sliced into 1-mm sections for radioactivity determinations. Data points have been omitted to facilitate ease in reproduction; only regions within and neighboring the histones and protein A24 are shown. - - - , \[^{3}\text{H} \text{ lysine radioactivity; } - - - - - , \[^{14}\text{C} \text{ arginine radioactivity.}

Fig. 4. Resolution of histones, protein A24, and non-histone proteins into Gaussian curves. A DuPont curve analyzer was used to fit Gaussian curves to the data of Fig. 3B. Solid lines are those generated by the curve resolver, while original data are shown as points.

Histone H1 Stoichiometry—Table I summarizes the results of stoichiometry estimates of the chromosomal protein patterns shown in Fig. 3; all molar ratios have been normalized relative to histone H4. Histones H1 (A + B) molar ratios were not determined from arginine content, since histone H1 species are known to contain only a few arginine residues, as opposed to a preponderance of lysine residues (36). Component X has been subtracted from all histone H1 data by curve shown in Fig. 3B; components X, Y, and Z are unknown protein bands which migrate near histones H1A, H3, and H2A, respectively. In spite of the fact that discontinuous data were used for curve-fitting, it is clear that an extremely accurate reflection of the proportions of individual protein species is obtained by curve analysis, as judged by the closeness of fit of the curves generated by summations of the Gaussian components to the original data points (Fig. 4). In most instances, other patterns of Fig. 3 were fitted equally well to individual Gaussian components (not shown). The reproducibility of curve fitting was tested by repeated analyses of the data of each sample. The areas of individual protein components of the same sample were found to vary between fits by 5 to 15%; larger errors being correlated with those samples which contained protein species less well separated (e.g. Fig. 3F).
It was, therefore, of interest to determine the relative proportions of these histone classes among different structure, as has been demonstrated for other histone H1 stoichiometry calculations from available sequence data (42).

It is likely that histones H1A and H1B of cultured mouse cells each contain several subfractions which differ in primary structure, as has been demonstrated for other histone H1 species (43, 44). It was, therefore, of interest to determine the relative proportions of these histone classes among different nucleoprotein species. As shown in Table I, the mole ratios of histone H1A/H1B are reduced significantly in mononucleosome fractions as compared to the mole ratios of these proteins in the other samples. This difference may be critically dependent on the position of the mononucleosome region which is excised from preparative gels, since earlier studies have shown mobility differences among mononucleosomes containing different forms of histone H1 (see Fig. 11 of Ref. 29). On the other hand, mononucleosomes which have histone H1A bound may be processed more rapidly to MI during micrococcal nuclease digestion, a condition known to be accompanied by the release of histone H1 from its resident binding sites (27, 29, 31, 45).

The data of Table I suggest that a substoichiometric amount (0.64) of histones H1 (A + B) exist per nucleosome in isolated nuclei of cultured mouse cells (that is, moles of H1 (A + B) per 2 mol of H4). This result could be explained by a number of complicating factors such as poor yield, proteolysis, labeling artifacts, substantially reduced numbers of lysine residues in histone H1 species of the cell line used, and overestimation of histone H4 content. However, we believe that these variables do not influence the general conclusion that histones H1 (A + B) are substoichiometric in isolated nuclei for the following reasons. (i) As discussed above, over 99.9% of the labeled protein enters the separating gel under the conditions employed. (ii) The cell line studied here grows in suspension culture and thus is not exposed to trypsin during transfer operations. This cell line contains extremely low endogenous chromatin protease activity, and a protease inhibitor (phenylmethylsulfonyl fluoride) has been used during preparative procedures. (iii) Radioactive lysine was maintained at constant specific activity in the medium during the entire 8-generation labeling period, and lysine incorporation data give stoichiometry estimates in accord with arginine incorporation data for the other histones (see below). (iv) Although a limited number of amino acid composition studies have been performed on histone H1 species of rapidly dividing cultured cells, the results of several reports indicate that the lysine content is similar to the value used in the present investigation for histone H1 stoichiometry calculations (46, 47). (v) Histone H4 shows symmetrical peaks of 3H and 14C radioactivities; it is unlikely that this protein has been overestimated by virtue of the co-migration of non-histone proteins as demonstrated by an analysis of isotope ratios (see below).

It is noteworthy that digested chromatin and isolated nuclei have the same content of histone H1 (Table I). Therefore, in spite of the knowledge that histone H1 is released from mononucleosomes during nuclease processing, no significant amount of histone H1 was lost during chromatin preparation following nuclear digestion. We conclude that released histone H1 most probably redistributes to oligonucleosomes during or following nuclear digestion; this view is in accord with other findings in the literature (48).

As shown in Table I, nucleoprotein species D6, D5, and D4 contain on average 1.76, 1.64, and 0.80 molecules of histones H1 (A + B) per dinucleosome, respectively (that is, moles of H1 (A + B) per 4 mol of H4). The observation that deviations from integral numbers of histone H1 molecules exist for dinucleosome classes suggests the possibility that other proteins may substitute for histone H1 species in a fraction (~20%) of the nucleoprotein complexes in creating similar electrophoretic mobility changes. Nevertheless, the observed histone H1 molar ratios are close to 2, 2, and 1 copies per D1.
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D0, and D1, respectively, and differ from the suggestion of Varshavsky et al. (27) that similar electrophoretic forms of dinucleosomes have 2, 1, and 0 copies of histone H1 molecules, respectively. However, this earlier suggestion was based on staining methods without knowledge of molar dye-binding coefficients, and rather low loads of proteins were used (27); these procedures are known to suffer potential pitfalls (12).

As is the case for dinucleosome classes, deviations from integral molar ratios exist for histone H1-containing mono- and dinucleosomes. Mononucleosome classes MIV-V, MIIL, and MI contain on average 0.72, 0.82, and <0.001 copies of histones H1 (A + B) molecules, respectively (that is, moles of H1 (A + B) per 2 mol of H4; see Table I). The absence of histone H1 species from MI is in agreement with previous reports (27–31). Although not studied here, histone H1 is absent also from MI (29–31).

The stoichiometry data described above were tested for internal consistency in the following way. The average number of histone H1 molecules per nucleosome in digested chromatin was reconstructed by a summation of the products of the individual stoichiometries and DNA mass fractions of each nucleoprotein species. Since stoichiometry values for 20% of the total DNA of digested chromatin were not determined (oligonucleosomes > 2N; see Fig. 2A), for purposes of calculation, a value of 1 histone H1 molecule/nucleosome was assigned to this chromatin fraction. The resulting value obtained by such a reconstruction process was 0.59 histone H1 molecule/nucleosome, which is in excellent agreement with the experimental value of 0.60 histone H1 molecule/nucleosome determined for digested chromatin (Table I).

To summarize the above findings, cultured mouse cell chromatin contains upper limits of 1 and 2 histone H1 molecules/mono- and dinucleosome class, respectively. However, isolated nuclei contain less than 1 copy of histone H1/nucleosome, and it is likely that histone H1 molecules which are released during nuclease digestion redistribute to oligonucleosomes which were originally deficient in these species. In particular, no dinucleosome class was found which lacked histone H1 and the nuclear content of histone H1 was recovered totally in digested chromatin. Renz et al. (48) have reported a binding preference of histone H1 for oligonucleosomes greater than pentamer in length at ionic strengths higher than 60 mM. However, the digestion conditions used in the present study preclude the existence of substantial amounts of larger oligonucleosomes. Therefore, our findings on dinucleosome histone H1 content are probably artifacts of protein redistribution. Germance is by this point the finding that dinucleosome class D1 which contains about 1 copy of histone H1, does not possess nucleosome cores with significant 160-base pair barriers toward nuclease digestion (31). Since mononucleosomes containing tightly bound histone H1 molecules are known to possess 160-base pair barriers (29, 31), this suggests that D1 contains redistributed histone H1 molecules which are bound in a nonphysiological manner. Therefore, the most likely arrangement of histone H1 molecules along nucleosomal arrays, prior to nuclease digestion-mediated protein redistribution, is either 1 copy or 0 copies of histone H1 molecules per histone octamer and not the more complex alternating possibility of 1 copy of histone H1 for every other histone octamer.

Core Histone Stoichiometry—Estimates of the molar ratios of histones H3, H2 (A + B), H2B, and H2A are shown in Table I for various chromatin subfractions. These values were calculated independently from both lysine and arginine content and are normalized relative to histone H4. Components Y and Z have been subtracted by curve analysis from data of histone H3 and histone H2A, respectively (Fig. 4). Among different samples, component Y was estimated to comprise between 0 and 11% of the total lysine or arginine content of the histone H3 region, while component Z was estimated to comprise from 1.7 to 8% of the total lysine or arginine content of the histones H2 (A + B) region.

As shown in Table I, the nucleosomal core histones are essentially equimolar among all chromatin fractions studied; a unit difference (0.5 mol/mol of H4) in a particular histone class does not exist in molar ratios between samples. Smaller than unit differences do exist in molar ratios between samples, but these can be attributed principally to experimental error based on our experience with the techniques employed in the present study. It is concluded, therefore, that electrophoretic fractionation of mono- and dinucleosomes is not due to differences in the molar ratios of the four smaller histone classes between nucleoprotein species. Given this conclusion, the data of each histone class have been averaged so as to permit a statistical evaluation. As shown in Table I, the overall means of core histone stoichiometries indicate that histone H3 is above unity (1.11 ± 0.11), histone H2B is close to unity (0.99 ± 0.06), and histones H2 (A + B) or histone H2A alone are below unity (0.90 ± 0.06 or 0.81 ± 0.09, respectively). These data were analyzed for statistical significance by a two-tailed t-test between a mean and a constant (H4 taken as 1.00; see Ref. 49). This analysis indicated that the stoichiometry of histone H2B does not differ significantly from unity (p > 0.2), while the molar ratios of histones H3, H2 (A + B), and H2A all differ from unity in a statistically significant manner (p < 0.002). Further statistical analyses of the overall means of core histone stoichiometries in all pairwise combinations indicated that all values were significantly different (p < 0.001; see Ref. 49).

The statistically significant differences between means discussed above could be due to inaccuracies in the resolution of histone species by curve analysis and could also be a result of overestimates of histone H4 and certain other histone classes due to the co-migration of non-histone proteins. Therefore, an estimate of the homogeneity of histone classes and the fidelity of curve fitting was performed by an isotope ratio analysis. The value C was calculated for each histone fraction analyzed from the following equation:

\[ C = \frac{\left[ ^{15}C \text{ cpm} \right]}{\left[ ^{14}H \text{ cpm} \right]} \]  

(number lysine residues)  

(number arginine residues)

The data were then normalized such that the sums of the C values of all histone classes studied in any given chromatin fraction were 1.00 (Table II). After performing this procedure, quick comparisons of data for deviations from unity can be made, either within protein classes between chromatin fractions or between protein classes within chromatin fractions. Using this approach, if a significant lysine- or arginine-rich non-histone protein(s) co-migrates with a given histone, then C for that histone class should be less than or greater than 1.00, respectively. Similarly, deviations from unity will exist for histone classes inadequately resolved by curve analysis. On the other hand, if all histones within a sample are homogeneous and resolved properly, or if given histones are contaminated with species which have a correspondingly similar number of lysine and arginine residues, then C will remain unchanged. It should be noted that such an analysis is independent of the molar ratios of the histones, but dependent on the agreement between isotope ratios and known lysine to arginine ratios of the various histones.

Table II shows the results of the isotope ratio analysis discussed above. It is noteworthy that the overall mean value of C for histone H4 is 1.00 ± 0.05, thus attesting validity to the use of histone H4 content to normalize the data shown in Table I. Furthermore, for histone classes H2 (A + B) and...
H2B, the overall mean values of C are not significantly different (p > 0.5) from the mean value of C of histone H4, as judged by a two-tailed t-test (49). Since a similar statistical analysis indicates that the value of C for histone H3 shows a significant (p < 0.02) enrichment in lysine content, it is reasonable to conclude that the stoichiometries of histone H3 and H4 are not significantly different. This conclusion was reached earlier (30). However, a statistically significant substoichiometric amount of histones H2 (A + B) alone are substoichiometric are supported strongly. In agreement with an earlier suggestion (30), mononucleosome class MI contains a greatly reduced content of protein A24 (Table I). At least three possibilities can be offered to explain this observation: (i) mononucleosomes which have protein A24 bound may migrate more slowly during electrophoresis; (ii) mononucleosomes which have protein A24 bound may be resistant to nuclease digestion; and (iii) protein A24 may be released from mononucleosomes during nuclease digestion. At present, insufficient data are available to decide between these alternatives.

Goldknopf et al. (25) have suggested that protein A24 replaces histone H2A in the core of the nucleosome. This proposal predicts that histone H2A should be substoichiometric in chromatin, a result borne out by the present investigation. A further prediction of this proposal is that nucleosomes lacking protein A24 should contain more histone H2A. Therefore, one might expect that mononucleosome class MI should contain an increased amount of histone H2A; clearly, more stoichiometry determinations are necessary before this possibility can be evaluated by a statistical analysis.

**Core Histone:DNA Mass Ratios**—The above results demonstrate that the molar ratios of the nucleosomal core histones are predominantly equimolar among electrophoretically fractionated mononucleosomes. Therefore, one would expect that dinucleosomes should contain essentially the same mass of nucleosomal core histones as do mononucleosomes, and that different electrophoretic forms of each of these components should contain nearly identical amounts of core histones. That dinucleosomes do contain by mass the amount of core histones as do mononucleosomes has been shown earlier for nucleoprotein species fractionated on sucrose gradients (15). However, in view of the knowledge that such...
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<th>Nucleoprotein species</th>
<th>Isotope ratios</th>
<th>Average molar mass relative to M1</th>
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<td></td>
<td>1.00 (base pairs)</td>
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<td>MI</td>
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<tr>
<td>MII</td>
<td>0.061 0.90</td>
<td>0.065 1.72 1.02</td>
</tr>
</tbody>
</table>

*Cells labeled with [3H]arginine and [3H]thymidine were used to prepare chromatin after 18% nuclear digestion. Nucleoprotein species which were separated electrophoretically as in Fig. 2A were excised, and portions were counted to determine isotope ratios after solubilization in NCS-scintillation mixture.

**From 5 to 15 x 10^6 cpm of the excited samples each were separated on 18% acrylamide, 0.1% SDS slab gels. Entire sample tracks were sliced and counted to determine the fractions of the total separated on 18% acrylamide, 0.1% SDS slab gels. Entire sample fractions were counted to determine isotope ratios after solubilization in NCS-scintillation mixture.

Addendum—After this manuscript was submitted for publication, we became aware of the findings of Zweidler (58) which indicate that mouse cells contain minor histones termed M1 and M2 with amino acid compositions strikingly similar to that of histone H2A. Inspection of Fig. 3 of Ref. 30 reveals that minor protein bands with mobilities corresponding to M1 and M2 in two different polyacrylamide gel systems uniformly are present among different electrophoretic forms of mono- and dinucleosomes of the cell line studied here. By densitometry, M1 and M2 comprise 7.3 ± 1.4% and 10.2 ± 1.0% (n = 6) of the mass of histone H2A of this cell line, respectively. We suggest, therefore, that in addition to protein A24, minor histones M1 and M2 substitute for histone H2A in the octamer protein core of the nucleosome, thereby bringing the observed submolar value of 0.91 for the sum of the stoichiometries of histone H2A and protein A24 approximately to equimolar (0.91 + 0.81) X (0.07 + 0.10) = 1.05 mol/mol of H4). Experiments employing Triton X-100 acid-urea gel electrophoresis (41) on cultured mouse cell acid-soluble chromatin proteins, followed by SDS-gel electrophoresis in the second dimension, demonstrate that M1 corresponds to component Z of Fig. 4, while M2 migrate between H3 and H2B (not shown).

REFERENCES

Histone Molar Ratios among Discrete Nucleoprotein Complexes

Histone molar ratios among different electrophoretic forms of mono- and dinucleosomes.
S C Albright, P P Nelson and W T Garrard


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