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Properties of Chromatin Subunits from Developing Trout Testis*

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SUMMARY

When a sample of trout testis nuclei is digested with micrococcal nuclease, the DNA is cleaved almost entirely to discrete fragments approximately 200 base pairs long and multiples thereof. The same DNA fragments can be obtained when isolated chromatin, as opposed to intact nuclei, is nuclease digested. These DNA fragments can also be found in discrete chromatin "subunits" isolated from nuclease-digested nuclei. Sedimentation through sucrose gradients or velocity sedimentation in an analytical ultracentrifuge separates these chromatin subunits into 11 S (monomer), 16 S (dimer), and 22 S (trimer) etc. species. Subunits can also be fractionated on a Sepharose 2B column equilibrated and run in low salt. High salt (>40 mM NaCl) or divalent cations (≃5 mM) cause subunit precipitation.

Chromatin subunits have a protein to DNA ratio of approximately 1.2 and contain all the histones, including the trout-specific histone T. There are, however, no detectable nonhistone chromosomal proteins. Mg^{2+} precipitates of the 11 S chromatin monomers, when pelleted, are thin and clear, while oligomer Mg^{2+} pellets are thick and white. This could reflect a more symmetrical or ordered packing of 11 S monomers, which are deficient in histone I. This histone may cross-link the larger oligomers, resulting in a disordered Mg^{2+} complex.

These results are consistent with the subunit model of chromatin structure, based on 200 base pair long regions of DNA associated with histones. These subunits would be separated by nuclease-sensitive DNA spacer regions and cross-linked by histone I.

Recent work suggests that the proteins of chromatin are organized in an ordered, repetitive manner along DNA. Hewish and Burgoyne (1) first reported that an endogenous nuclease in rat liver nuclei cleaved the DNA to multiples of a unit length. This suggested to them a basic repeating structure for chromatin. Noll (2) then found that treatment of intact rat liver nuclei with the endogenous nuclease or commercially available micrococcal nuclease cleaved 85% of the DNA to fragments 200 base pairs long or multiples thereof. Louie (3) obtained similar results with polyoma nucleoprotein. Noll (2) could also isolate, from nuclease-treated nuclei, intact 11.2 S "chromatin subunits," containing histones and nonhistone proteins attached to stretches of DNA 200 base pairs long. Electron micrographs showing bead-like regions spaced along chromatin (4) support such a picture of subunits of nuclease-resistant, protein-bound DNA alternating with nuclease-sensitive, DNA spacer regions. Kornberg (5, 6) has extended such results, along with his own data on cross-linked histone oligomers, into a theory of chromatin structure based on a repeating unit of 200 DNA base pairs and two of each of the histones (except for histone I).

Noll (7) has observed that when chromatin *in situ* is digested with DNase I, a regular series of single-stranded DNA fragments, multiples of 10 bases long, is obtained. His work strongly suggests that the DNA is accessible to DNase, and therefore on the outside of the chromatin subunit, the structure of which contains some internal repetitive elements. Different DNA fragments, smaller than 200 base pairs long, have been reported when isolated chromatin, as opposed to intact nuclei, is treated with micrococcal nuclease (8–11). Such results also point to some regular repeat structure for chromatin, based on "specific contacts between protein and nucleic acid which arise from structural properties of the histones" (10). This may involve the cross-linking of DNA by the exposed NH_2 termini of a trypsin-resistant histone complex (11), but it is difficult to relate these nuclease digestion products to those obtained by Noll (2).

In a previous communication (12) we reported that micrococcal nuclease treatment of trout testis nuclei gave rise to 11 S chromatin particles containing a unit length of DNA and associated histones. This result was obtained in both mitotic and meiotic tissue but not in nucleoprotamine stage tissue. Because large amounts of nuclei are readily prepared from trout testis, it was of interest to characterize the 11 S chromatin particles from this source and to examine the DNA products of micrococcal nuclease digestion of nuclei *versus* isolated chromatin. The results lend further evidence to a subunit model (1, 2, 6) for chromatin structure, based on 200 base pair long stretches of DNA and associated histones.

MATERIALS AND METHODS

Preparation and Digestion of Nuclei or Isolated Chromatin—Nuclei were prepared from naturally maturing trout testis (Sun

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Valley Trout Farm, Mission, B. C.) in 50 mM Tris, pH 7.4, 25 mM KCl, 1 mM $MgCl_2$, 0.25 M sucrose, and 15 mM 2-mercaptoethanol (Buffer A) buffer as previously described (12). Washed nuclei were then incubated with 500 A_{260} units/ml of micrococcal nuclease (Sigma) at 37° in Buffer A-1 mM $CaCl_2$ for 1 to 10 min. Chromatin was prepared by washing nuclei once in 0.15 M NaCl-0.02 M EDTA, then three times in 10 mM Tris, pH 8.0. Digestion of chromatin (20 A_{260}/ml) was performed at 37° in 10 mM Tris, 0.1 mM $CaCl_2$, and 300 units/ml of micrococcal nuclease.

Preparation of "Chromatin Subunits" from Digested Nuclei—After treatment with micrococcal nuclease, nuclei were collected at 1,000 × *g*, resuspended vigorously in 0.2 mM EDTA, and spun 30 min at 12,000 × *g*. The supernatant, containing subunits (10 to 200 A_{260}/ml), was then layered on a 10 to 30% sucrose-0.2 mM EDTA gradient (12 ml) and spun at 36,000 rpm for 12 to 14 hours in a Beckman SW 40-Ti rotor. Fractions of 0.4 ml were collected by puncturing the gradient tube, followed by upward displacement of the gradient with 60% sucrose.

Sepharose 2B Chromatography—The 0.2 mM EDTA supernatant, containing chromatin subunits (10 to 100 A_{260}/ml), was loaded onto a Sepharose 2B (Pharmacia) column (2.5 × 40 cm) equilibrated and run in 5 mM Tris-0.2 mM EDTA, pH 7.6.

Analysis of DNA Fragments—DNA was prepared from micrococcal nuclease-treated nuclei or chromatin or from isolated subunits by phenol extraction (1). The resultant fragments were analyzed on 2.5% polyacrylamide gels (2, 13) or on denaturing 99% formamide/6% polyacrylamide gels (7, 14), essentially as described.

Quantitation of Subunit Proteins—Aliquots of subunits, either before or after sucrose gradient centrifugation, were taken and made 1 to 5% in sodium dodecyl sulfate, heated to 100°, then made 20% in sucrose (w/v), 10 mM in Tris, pH 7.2, 0.15 M in 2-mercaptoethanol and were subjected to electrophoresis on 5% sodium dodecyl sulfate polyacrylamide gels (15). On these gels, histone I migrates as a discrete band behind the rest of the histones, which migrate together as a single band near the dye marker. Subunits were sometimes treated with DNase I (5 μg/sample) prior to sodium dodecyl sulfate treatment.

Alternatively (*a*) subunits were first precipitated with 10 mM Mg^{2+} (12), then heated to 100° in sodium dodecyl sulfate-Tris-sucrose-2-mercaptoethanol loading buffer before gel electrophoresis; or (*b*) subunits were precipitated with Mg^{2+}, then acid extracted with 0.2 N HCl. The supernatant was diluted with water and lyophilized, and the resulting proteins (histones) subjected to electrophoresis as above on 5% sodium dodecyl sulfate or urea (16) polyacrylamide gels. Gels were stained for protein with Coomassie blue and scanned at 550 nm in a Gilford spectrophotometer. 0.2 N HCl extracts of subunits were also subjected to electrophoresis on urea-lactate gels as previously described (20). Non-histone chromosomal proteins were prepared from nuclei or chromatin samples according to the method of LeStourgeon and Rusch (17) or by direct hot 5% sodium dodecyl sulfate extraction. Protein determinations on histone samples were performed by trichloroacetic acid turbidity (18) or A_{230} measurements (18), using isolated trout testis histones as standards.

Sedimentation Experiments—These were done with a Beckman model E ultracentrifuge at 36,000 rpm, 4°, using scanner optics at 260 nm, on 0.8 A_{260}/ml of subunits in 20 mM Tris-0.2 mM EDTA, pH 7.0. Alternatively, subunits (60 A_{260}/ml) were sedimented using Schlieren optics. $s_{20,w}$ values were calculated for chromatin subunits using a partial specific volume of 0.69 for chromatin (9).

RESULTS

Chromatin was isolated from nuclei digested briefly (2 min) with 500 units/ml of micrococcal nuclease and was fractionated on a sucrose gradient. The resultant profile (Fig. 1) shows a main peak (at ≃11 S), a second peak (at ≃16 S), and a shoulder of heavier material. Characterization on 2.5% polyacrylamide gels of the DNA extracted from fractions of the gradient (photos in Fig. 1) shows that the main 11 S peak contains a small DNA fragment, while the second peak contains predominantly a larger DNA piece, and the shoulder region has even larger DNA fragments.

Noll (2) has calibrated the mobilities of such DNA fragments

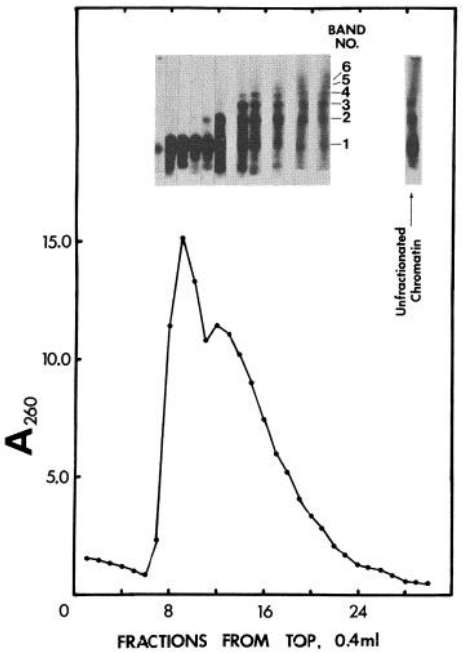

FIG. 1. Characterization of DNA in chromatin from digested nuclei. Chromatin was isolated from nuclei digested with micrococcal nuclease and was fractionated on a 5 to 30% sucrose gradient as described under "Materials and Methods." Material from the gradient fractions was precipitated with 10 mM Mg^{2+}, resuspended in 1% sodium dodecyl sulfate, 20 mM EDTA, and 1 M NaCl, and was extracted with phenol. DNA was precipitated with 2 volumes of ethanol and analyzed on 2.5% polyacrylamide gels. Gels were stained with "Stains-All"; gel photos appear above corresponding gradient fractions.

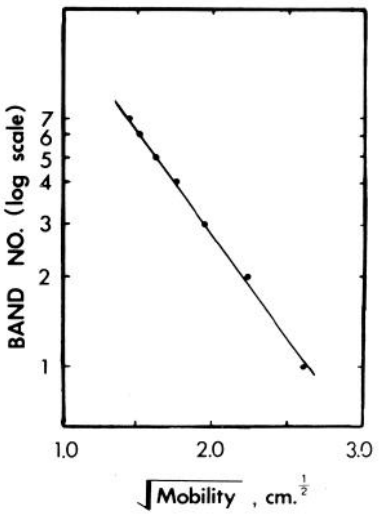

FIG. 2. Polyacrylamide gels (2.5%) of DNA fragments from digested nuclei. Plot of DNA band number against square root of band mobility. Data is taken from the gels in Fig. 1.

(from rat liver nuclei) with the same 2.5% polyacrylamide gel system against sequenced DNA markers. He provided good evidence that the fragments so produced are 205 ± 15 base pairs long and multiples (approximately 400, 600 *etc.* base pairs) thereof. A plot of log DNA band number *versus* square root of band mobility for these fragments was linear (1, 19).

A plot of log DNA band number *versus* square root of mobility is also linear for the DNA fragments from trout testis (Fig. 2), indicating that the larger DNA fragments are approximately integral multiples in length of the smallest DNA piece. We esti-

mate a single-stranded length of roughly 200 ± 30 nucleotides for this smallest trout DNA piece, using polyacrylamide gel electrophoresis in 99% formamide (14) against Drosophila 5 S and tRNA markers and based on Noll's (7) careful calibration of RNA and DNA mobilities in this system.

Closer examination of the smallest trout DNA fragment shows that it consists of two species corresponding to monomer, 200 ± 30 base pairs, and a smaller, partially cleaved monomer DNA, 175 ± 25 base pairs (Fig. 3*a*, *bands 1* and *1a*). Noll (2) has estimated a length of 170 ± 10 base pairs for this cleaved monomer from rat liver nuclei. On overloaded DNA gels (Fig. 1, gels across the 11 S peak) two even smaller DNA fragments appear, but in extremely low yield (*cf.* the unfractionated chromatin sample in Fig. 1).

When isolated chromatin, as opposed to intact nuclei, is digested with micrococcal nuclease for 1 to 10 min, the same DNA fragments can be isolated and characterized on 2.5% polyacrylamide gels (Fig. 3*b*) or as single-stranded fragments on 99% formamide/6% polyacrylamide gels. Clearly here (Fig. 3*b*) the small molecular weight fraction consists of two species, monomer (*band 1*) and "trimmed" monomer (*band 1a*). Extended (1 hour) digestion of isolated chromatin results in the appearance of DNA fragments much smaller than 200 base pairs long. Axel *et al.* (10) and Weintraub and Van Lente (11) have examined the fragments from such extended chromatin digests and find pieces 45 to 130 base pairs long, which presumably reflect nuclease cleavage sites within a subunit monomer.

Other estimates of chromatin subunit size come from sedimentation velocity experiments on the Beckman model E ultracentrifuge. Chromatin from digested nuclei separates (Fig. 4) into a major peak at 11.4 S (monomer), a second peak at 16 S (dimer), and a third at 22 S (trimer). The symmetry of the peaks argues strongly for homogeneity in size, if not composition. These size estimates correlate well with those obtained on sucrose gradients; however, the ion concentration present (20 mM Tris-HCl) may have been insufficient to prevent anomalies due to salt effects. At lower salt (0.2 mM EDTA) or subunit concentrations (0.8 A_{260}/ml), S values were slightly lower (10.5 S for the monomer).

Monovalent cations (Na^+, K^+, Tris) effected 50% precipitation of subunits at concentrations around 70 mM, while divalent cations (Mg^{2+}, Ca^{2+}) precipitated 50% of the subunits near 1 mM (Fig. 5), Ca^{2+} being slightly more effective than Mg^{2+}.

Chromatin subunits could also be roughly fractionated on a Sepharose 2B column (Fig. 6), again in low salt. Monomers

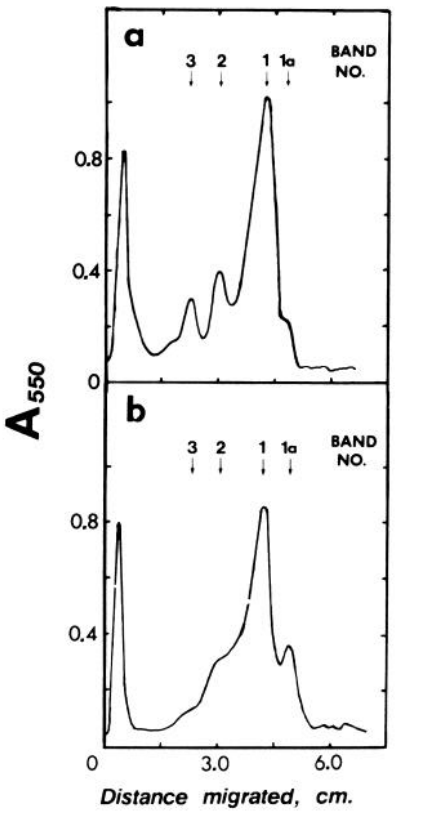

FIG. 3. Characterization of DNA fragments (*a*) from intact nuclei and (*b*) from isolated chromatin, digested with micrococcal nuclease. Intact nuclei (*a*) or isolated chromatin (*b*) were digested with micrococcal nuclease under identical conditions as described under "Materials and Methods." DNA was prepared by phenol extraction and ethanol precipitation and was analyzed on 2.5% polyacrylamide gels. Gels were stained in "Stains-All" and scanned at 550 nm in a Gilford spectrophotometer.

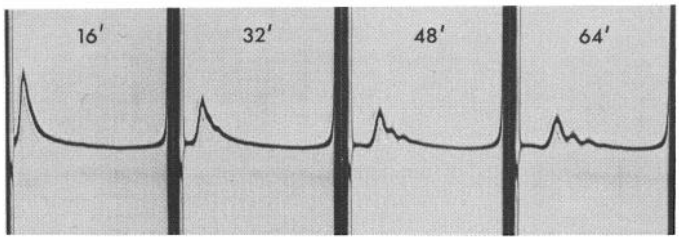

FIG. 4. Sedimentation velocity analysis of chromatin from digested nuclei. Chromatin (60 A_{260}/ml of DNA) was isolated from nuclei digested with micrococcal nuclease and was sedimented in a single sector cell at 36,000 rpm, 5°, in 20 mM Tris, pH 7.6, and 0.2 mM EDTA, on a Beckman model E analytical ultracentrifuge using Schlieren optics. Photos were taken at 16-min intervals during the run, and $s_{20,w}$ values were calculated as described under "Materials and Methods." The phase plate angle was 50°.

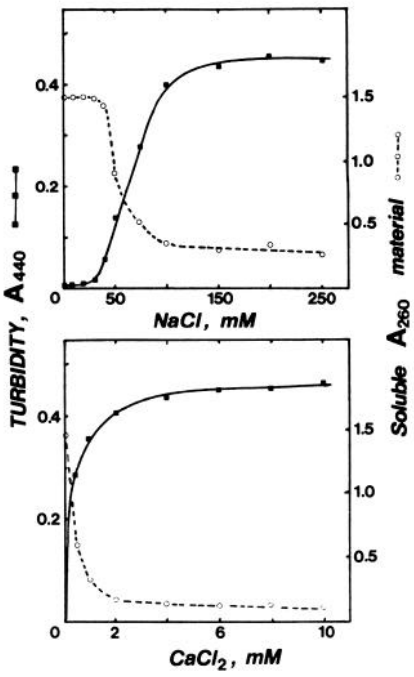

FIG. 5. Precipitation of chromatin subunits by monovalent and divalent cations. Chromatin subunits (1.5 A_{260}/ml) were treated with varying concentrations of (*a*) NaCl, Tris-HCl, or (*b*) $CaCl_2$, $MgCl_2$, and turbidity at 440 nm was monitored. Then samples were centrifuged at 4000 rpm for 10 min and supernatant absorbance at 260 nm was measured.

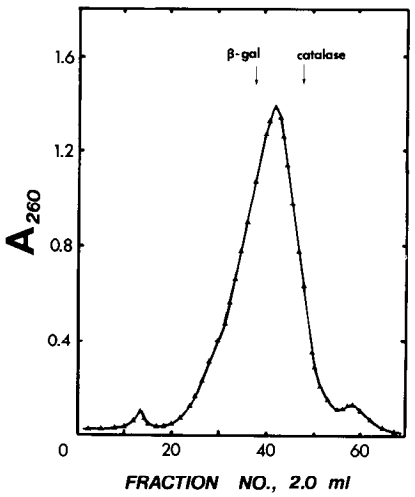

FIG. 6. Sepharose 2B chromatography of chromatin isolated from nuclease-digested nuclei. Chromatin (10 to 100 A_{260}/ml of DNA) from nuclease-digested nuclei was loaded on a Sepharose 2B column (2.5 × 40 cm) equilibrated and run in 0.2 mM EDTA and 5 mM Tris, pH 7.6. Fractions of 2.0 ml were collected and A_{260} was monitored. The approximate elution positions of *Escherichia coli* β-galactosidase (M_r = 540,000) and bovine catalase (M_r = 240,000) are marked by *arrows*.

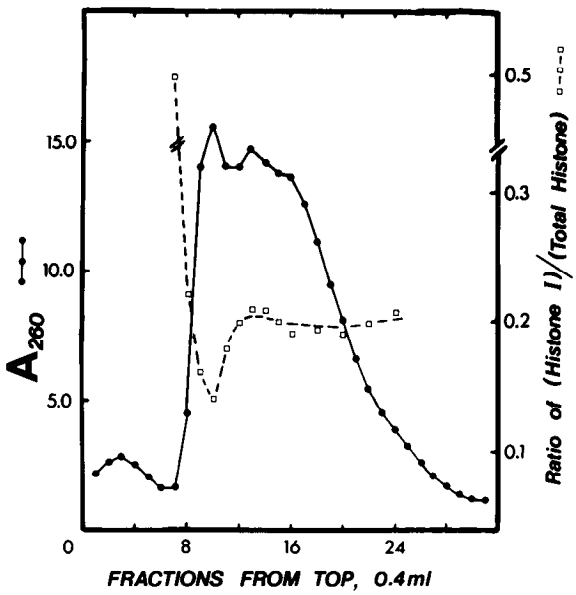

FIG. 7. Histone content in chromatin from digested nuclei, fractionated on a sucrose gradient. Chromatin was isolated from nuclei digested with micrococcal nuclease and was fractionated on a 5 to 30% sucrose gradient. Proteins were extracted in 2% sodium dodecyl sulfate from Mg^{2+} precipitates of gradient fractions and were subjected to electrophoresis on sodium dodecyl sulfate 5% polyacrylamide gels. Gels were stained with Coomassie blue and scanned at 550 nm on a Gilford spectrophotometer. Histone I and total histone were quantitated from scan areas.

eluted in a position corresponding to a molecular weight of approximately 400,000. A similar result was obtained with Sepharose 4B. This is slightly higher than expected for an 11 S particle containing 200 base pairs of DNA and an approximately equal weight of histones. This may indicate deviation of the subunits from a spherical shape or a large hydration shell for the presumably highly charged subunits.

The protein complement of these subunits was next characterized. They showed a protein to DNA ratio of approximately 1.2 and an A_{230}:A_{258} ratio of 0.72, both lower than values (1.5 and 0.8, respectively) reported for nuclease digests of isolated chromatin (9).

The five major histone species, as well as the trout-specific histone T, could be identified, but no non-histone proteins were detectable on sodium dodecyl sulfate polyacrylamide gels even when heavily overloaded for histones.

Across a sucrose gradient fractionation of chromatin subunits, it was noteworthy that Mg^{2+} pellets for the monomer fractions (Fig. 7, *Fractions 8* to *10*) were thin and clear, whereas the oligomer fractions gave opaque white pellets. The monomer peak was also relatively deficient in histone I (Fig. 7), with an excess of free histone I appearing at the top of the gradient. The deficiency of histone I in subunit monomers is even more dramatic when extensive digests containing little oligomer are examined. The histone I to total histone ratio of 0.2 obtained from Coomassie blue staining on gels is somewhat high, since the larger histone I binds proportionately more dye.

The histones (except histone I) of chromatin subunits are present in roughly the same proportions as in whole chromatin, *i.e.* roughly equimolar, as judged by polyacrylamide (16) and starch gel (20) electrophoresis.

DISCUSSION

Our results show that micrococcal nuclease digestion of intact nuclei gives rise to 11 S chromatin particles (monomers) containing approximately 200 base pair long fragments of DNA and associated histones. Presumptive chromatin dimers (16 S) and trimers (22 S) resulting from incomplete digestion could also be isolated, containing correspondingly longer DNA pieces. The same DNA pieces, multiples of approximately 200 base pairs long as well as "trimmed monomer pieces," can be obtained following digestion of isolated chromatin from trout testis.

We obtain DNA fragments smaller than about 200 base pairs long only in extremely low yield after digestion of nuclei. These apparently reflect cleavage at additional sites within, as opposed to between chromatin subunits. Clark and Felsenfeld (8) and Sahasrabuddhe and Van Holde (9), using extended (up to 1 hour) nuclease digestion of isolated chromatin, reported that the DNA was digested to 100 base pair long fragments. Refinement of the analysis of digestion products by Axel *et al.* (10) and Weintraub and Van Lente (11) showed them to be discrete fragments 45 to 130 base pairs long, separated by 10 to 15 base pair intervals. Such fragments presumably reflect internal cleavage within a chromatin subunit monomer, although they may be derived from a minor nuclease activity or chromatin instability following prolonged incubation at 37° in low ionic strength. How these discrete DNA fragments are derived from the 200 base pair monomer is unclear, but they presumably reflect some internal asymmetry in the chromatin monomer.

These fragments may be related to those obtained by Noll's (7) elegant work with DNase I, which digests DNA into discrete pieces from 10 to 200 nucleotides long at 10 nucleotide intervals. Noll's work (7) strongly suggests that the DNA in a chromatin subunit is wound around the outside of a histone core, with some repetitive structural feature within the subunit.

The work of Axel *et al.* (10) and Weintraub and Van Lente (1) suggests that the structure of the DNA-protein complex is determined by DNA cross-links with a histone core resulting in nuclease-resistant DNA regions. However, in view of the presumably intricate mechanisms involved in the assembly of chromatin (21), it is surprising that the same 45 to 130 base pair long fragments are obtained by Axel *et al.* (10) after reconstitution of chromosomal proteins with DNA from any source.

From the size of the DNA (200 base pairs ≃130,000 daltons), the S value (11 S), and the protein to DNA ratio (1.2:1), eight to nine histone molecules would be expected per chromatin monomer. The fact that the histones appear to be roughly equimolar in chromatin subunits would argue for the presence of two of each of the major histones (except histone I) as was suggested by Kornberg (6). Heterogeneity with respect to which species of histone are present on individual chromatin subunits is not ruled out however. Protein cross-linking results would be extremely useful in obtaining a crude topographical map of the chromatin subunit, as has been done with ribosomes (22).

We could find no detectable non-histone proteins in trout testis chromatin either by the method of LeStourgeon and Rusch (17) or by direct hot 5% sodium dodecyl sulfate extraction. A few very minor protein species could be observed if washed nuclei, rather than chromatin, were used as starting material. It seems likely then that the non-histone proteins observed by some workers at total levels approaching equal weight with the histones could be nuclear proteins not associated with chromatin, *e.g.* from membranes (23) or cytoplasmic contaminants. Alternatively, the special nature of trout testis, a tissue "shutting down" genetic functions for packaging of DNA and protamine into sperm, may account for the lack of observable non-histone proteins.

The relative absence of histone I in chromatin monomers, as opposed to oligomers, coincides with the thin clear Mg^{2+} pellets from these preparations. This may reflect some symmetry (*e.g.* DNA with eight histones in a regular structure), allowing an ordered packing of monomers. These monomer pellets may prove useful in physical studies of subunit structure. The presence of histone I in oligomers may reflect the role of this histone in cross-linking (24) individual monomers, conferring asymmetry and resulting in a disordered complex in the presence of Mg^{2+}.

The cross-linking of free subunits by histone I may be the reason for the presence of small amounts of monomer DNA even in the larger chromatin fragments (Fig. 1), although this may reflect aggregation during gradient centrifugation. Histone-histone interactions alone are evidently not sufficient to hold the majority of monomer subunits together, as noted by Noll (2), as most monomer DNA runs in the 11 S monomer region. The continuity of the chain of chromatin subunits is therefore provided by its DNA.

The availability of large amounts of readily prepared nuclei from trout testis has greatly facilitated these studies. We now hope to use chromatin subunits in the study of histone modifications as related to chromatin assembly and structure.

Preliminary experiments[1] involving the incubation of cells with [*methyl*-^{14}C]methionine to label histones on chromatin indicate that histones already assembled into subunits are methylated. This correlates well with the observation that methylation is a relatively late event in the cell cycle[2] (25), occurring after histone and DNA synthesis. [^{14}C]Acetate label in histones, however, appears not in subunits but in free histones (at the top of sucrose gradients), which is consistent with data indicating that histones must first be processed (*e.g.* acetylated and deacetylated) before assembly into chromatin (21).

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