Chromosomal Proteins of *Drosophila* Embryos†

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ABSTRACT: Because of the wealth of genetic information and mutants available, and because it possesses polytene chromosomes during some developmental stages, *Drosophila* is being used increasingly in the study of the structure and function of the eukaryotic genome. We present here methods for the preparation of purified nuclei, chromatin, and chromosomal proteins from *Drosophila melanogaster* embryos and tissue culture cells. These methods have been used to prepare chromatin from different stages of embryonic development in *Drosophila*. Chromatin of the blastula (0–2-hr embryos) differs significantly from that of older embryos (6–18 hr) in chemical composition, nonhistone chromosomal protein pattern (as shown by sodium dodecyl sulfate gel electrophoresis), melting properties, and template activity. Blastula chromatin contains normal histones, except for a possible quantitative deficiency in histone I. Several alterations in the nonhistone chromosomal protein pattern are noted; in particular, the blastula chromatin contains a dominant protein band of mol wt 45,000 not apparent in the older embryo chromatin. It is suggested that the presence of this protein as a major component of the chromatin may be a consequence of the very rapid rate of nuclear division, and hence very high concentration of replication forks, in the early embryos.

There is currently widespread interest in the structure and organization of the eukaryotic genome and in the functional interaction of its macromolecular constituents. Research in several laboratories has demonstrated that the interphase form, chromatin, can be isolated for study *in vitro* using biochemically gentle techniques (Bonner *et al.*, 1968a); the isolated chromatin retains its capability as a template for tissue and stage specific transcription, to the extent that this has been tested (Paul and Gilmour, 1968; Bekhor *et al.*, 1969; Smith *et al.*, 1969; Chetsanga *et al.*, 1970; Axel *et al.*, 1973). The chromatin is a complex of DNA, RNA, histone, and nonhistone chromosomal proteins.[1] The DNA template itself is now known to be fairly complex, including repetitious sequences of different frequency and function (some organizational, some transcribed), as well as unique protein-coding sequences (reviewed by Rae, 1972). The histones, small basic proteins, are known to be able to inhibit transcription; they probably play a general structural role as well as being general repressors of template activity (Shih and Bonner, 1970; Smart and Bonner, 1971a,b; reviewed by Elgin *et al.*, 1971). Little is known about the NHC proteins; *a priori*, this fraction is thought to include enzymes of chromosomal metabolism, structural proteins, and possibly gene activity and/or repression signals. The RNA associated with chromatin is to some extent nascent messenger and includes small nuclear RNAs (Dahmus and McConnell, 1969; Busch *et al.*, 1972). Changes in the subpopulations and in the interactions of the macromolecules with DNA and each other no doubt account for changes in gene expression. Likewise, these associated macromolecules must be responsible, at least in part, for alternative means of packaging the genome—those seen in interphase, polyteny, metaphase, etc. Consequently, it is of interest to carry out detailed chemical studies of these macromolecules as isolated from chromatin of different states.

The wealth of data available on *Drosophila* suggests that it is the creature of choice for studies of chromatin for several reasons. Salient among these is the fact that the genetics of *Drosophila* are better known than those of any other eukaryote. This will be of vital importance when work advances to the stage where biological controls are needed and mutants can be exploited, as they have been in studying the lac system in prokaryotes (Epstein and Beckwith, 1968). *Drosophila* is also one of the few eukaryotic organisms in which polytene chromosomes can readily be observed; such chromosomes present a unique opportunity for studies of gene activation and transcription, as demonstrated by the recent work of Ashburner (1972), Daneholt (1972), and others. In this paper we will demonstrate that one can readily prepare large amounts of chromatin from *Drosophila* embryos or tissue culture cells, and present biological and chemical characteristics of this chromatin. The techniques will then be used to examine chromatin from *Drosophila* blastula, an interesting developmental stage showing very rapid nuclear division.

Materials and Methods

Drosophila. Large quantities of *Drosophila melanogaster* (Oregon R) embryos can be harvested using grape juice–agar plates as developed by D. S. Hogness, W. J. Peacock, and colleagues (personal communication). The eggs are washed off the plates with saline solution, washed thoroughly over nylon filters, and stored frozen at −80°. An adult population of 200,000–300,000 flies will produce *ca.* 30–60 g of eggs per day. Embryo collections are designated according to their age at harvest in hours. Immediately prior to use the embryos are thawed to ice temperature and the chorion removed by treatment with half-strength Clorox for 2 min at ambient temperature. The embryos are washed thoroughly with water on a nylon filter, washed with 70% alcohol followed by more water,

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[1] Abbreviations used are: NHC proteins, nonhistone chromosomal proteins.

then washed three times by settling in ice-cold saline (0.9% NaCl)–0.1% Triton X-100, and finally washed with water again (Kram *et al.*, 1972; A. Zweidler, L. H. Cohen, and A. Mahowald, personal communication).

Tissue culture cells (the generous gift of A. Blumenthal, Stanford University) were Schneider's line 2 grown in suspension in Schneider's medium (Schneider, 1972). The cells have a doubling time of 18–24 hr. They have a normal diploid karyotype, XX. Following harvest the cells were frozen in medium plus 10% glycerol and stored at −80° until use.

Preparation of Drosophila Embryo Chromatin. These methods are essentially modifications of the procedure of Marushige and Bonner (1966). All operations are carried out at 0–4°. Initial homogenization and filtration are done in a cold room.

METHOD 1. Dechorinated *Drosophila* embryos (40 g) are homogenized (Dounce A homogenizer) in 500 ml of buffer I (0.05 M Tris-HCl (pH 7.6)–0.025 M KCl–0.005 M MgOAc–0.35 M sucrose) (Ritossa and Spiegelman, 1965) and filtered gently through two layers of Miracloth (Chicopee Mills, Inc.). The crude nuclear pellet is collected by centrifugation at 750*g* for 10 min. The crude nuclei are washed once by resuspension in saline–EDTA (0.075 M NaCl–0.024 M Na_2EDTA (pH 8)) and collected by centrifugation at 1500*g* for 10 min. The nuclei are then lysed by vigorous resuspension in 0.01 M Tris (pH 8) and the crude chromatin is pelleted at 6000*g* for 10 min. The chromatin is washed twice more, with 0.005 and 0.001 M Tris (pH 8), respectively, and collected by centrifugation at 12,000*g* for 10 min. Care should be taken to ensure a smooth resuspension of the crude chromatin at each step. A Dounce homogenizer with pestle A may be used for resuspension at the 0.005 M Tris wash, but in general yields are better if resuspension is accomplished by vigorous stirring with a glass rod. The crude chromatin is then resuspended, using a glass–Teflon homogenizer, in 0.005 M Tris (pH 8) and is purified by centrifugation through 1.7 M sucrose (buffered with 0.005 M Tris (pH 8)) for 3.0 hr at 50,000*g*. The purified chromatin is washed twice with 0.01 M Tris (pH 8), sheared on a Virtis homogenizer at 30 V for 90 sec to make it soluble, and centrifuged at 12,000*g* for 30 min. The supernatant, purified soluble chromatin, is used immediately as chromatin or for purification of chromatin components. This purified chromatin has an ultraviolet (uv) absorbance spectrum such that $A_{230}/A_{260} < 1.0$ and contains no very basic proteins other than histones.

METHOD 2. Dechorinated *Drosophila* embryos (40 g) are homogenized in a Dounce homogenizer, pestle A, in 250 ml of buffer II (0.05 M Tris-maleate (pH 7.4)–0.005 M $MgCl_2$–0.01 M β-mercaptoethanol). The homogenate is filtered gently through two layers of Miracloth. The nuclei are collected by centrifugation at 2000*g* for 10 min (swinging bucket rotor) into a cushion of one-third volume of buffer II + 0.2 M sucrose. The crude nuclei are washed by resuspension and centrifugation at 2000*g* for 10 min in buffer II, buffer II plus 0.1% Triton X-100, and buffer II (A. Zweidler, L. H. Cohen, and A. Mahowald, personal communication). The nuclei are then resuspended in saline–EDTA and chromatin is prepared as given above.

Preparation of Drosophila Chromatin from Tissue Culture Cells. The frozen cells (see above) are thawed quickly (37°) and collected by centrifugation at 1500*g* for 10 min. The cells are then resuspended in saline–EDTA (pH 6.8). Triton X-100 (10%) is added to a final concentration of 0.5% and the solution stirred gently and left on ice for 10 min. Almost all the cells are lysed by this procedure. The nuclei are collected by centrifugation at 1500*g* for 10 min, washed once with saline–EDTA (pH 6.8), and then used for the preparation of chromatin as given above.

Preparation of Purified Drosophila Embryo Nuclei. Crude *Drosophila* embryo nuclei are prepared as given under method 2 above. Following the last wash with buffer II, the nuclei are resuspended in buffer II plus 0.2 M sucrose and layered over an equal volume of buffered 2.2 M sucrose (0.01 M Tris-maleate (pH 7.4)–0.005 M $MgCl_2$–0.01 M β-mercaptoethanol). The nuclei are pelleted by centrifugation at 70,000*g* (Spinco 25.2 rotor, 24K) for 100 min. The purified nuclear pellet may be resuspended in saline–EDTA and chromatin prepared as given above. By examination using phase-contrast microscopy and Feulgen staining, nuclei prepared by this method from 6–18-hr embryos are >98% pure, while those from 0–2-hr embryos are only *ca.* 70–80% pure.

Preparation of Chromosomal Proteins. The chromosomal proteins may be prepared by a combination of the methods of Elgin and Bonner (1970) and Dingman and Sporn (1964). Two volumes of cold 5 N NaCl are added to three volumes of chromatin (concentration 0.5 mg of DNA/ml or less) with rapid stirring. The mixture is allowed to flocculate 1 hr at 4°; then 1.25 vol of ice-cold 1 N HCl is added with rapid stirring. Following a second hour of flocculation at 4°, the pellet (consisting of DNA and NHC proteins) is collected by centrifugation at 30,000*g* for 20 min. The supernatant, containing the histones, is dialyzed extensively against 0.01 M acetic acid, and the proteins are recovered by lyophilization. The pellet is washed briefly with 0.01 M Tris (pH 8) and then dissolved in 1% sodium dodecyl sulfate–0.05 M Tris (pH 8). Following dialysis to 0.1% sodium dodecyl sulfate–0.01 M Tris (pH 8), the DNA is removed by centrifugation at 200,000*g* for 24 hr at 25°. The supernatant contains the NHC proteins; following dialysis to the appropriate buffer, these may be analyzed by disc gel electrophoresis and by chemical techniques.

Preparation of Drosophila DNA. DNA was prepared from purified sheared chromatin (see above) by dissociation of the chromatin with 1% sodium dodecyl sulfate plus 1 M LiCl followed by phenol extraction. Following reextraction of both phases, the combined aqueous phases were extracted twice with chloroform–isoamyl alcohol (24:1). The DNA solution was then made 0.15 M in NaCl and the DNA wound out on addition of 3 vol of ice-cold 95% ethanol. After dissolving in 0.01 M Tris (pH 8) the DNA was treated with bovine pancreatic RNase (Sigma, 5× recrystallized, preboiled 10 min) at 50 μg/ml for 60 min at 37°, and subsequently with Pronase (Calbiochem grade A, preincubated for 90 min at 37°) at 200 μg/ml for 30 min at 37°. The DNA was then reextracted twice with phenol and twice with chloroform–isoamyl alcohol, wound out from ice-cold ethanol as above, dissolved in 0.01 M Tris, and dialyzed extensively (Dahmus and McConnell, 1969). The product $A_{280}/A_{260} = 0.52$ and $A_{230}/A_{260} = 0.41$; its mol wt was $\sim 2 \times 10^6$ (sedimentation analysis).

Disc Gel Electrophoresis. The NHC proteins were analyzed by sodium dodecyl sulfate disc gel electrophoresis (molecular weight sieving) using either the phosphate buffer system of Shapiro *et al.* (1967) or the Tris-glycine buffer system of Laemmli (1970) as previously described (Wu *et al.*, 1973). Histones were analyzed on 15% polyacrylamide gels (pH 4.3) in the presence of urea (Bonner *et al.*, 1968a).

Chemical Analysis of Chromatin. The concentration of DNA in a given sample of chromatin was determined from the optical absorptivity at 260 mμ (corrected for scattering) assuming $A = 21.6$ l./(cm g) and allowing for the RNA contribution of $A = 25.0$ l./(cm g) (Tuan, 1967). RNA concentra-

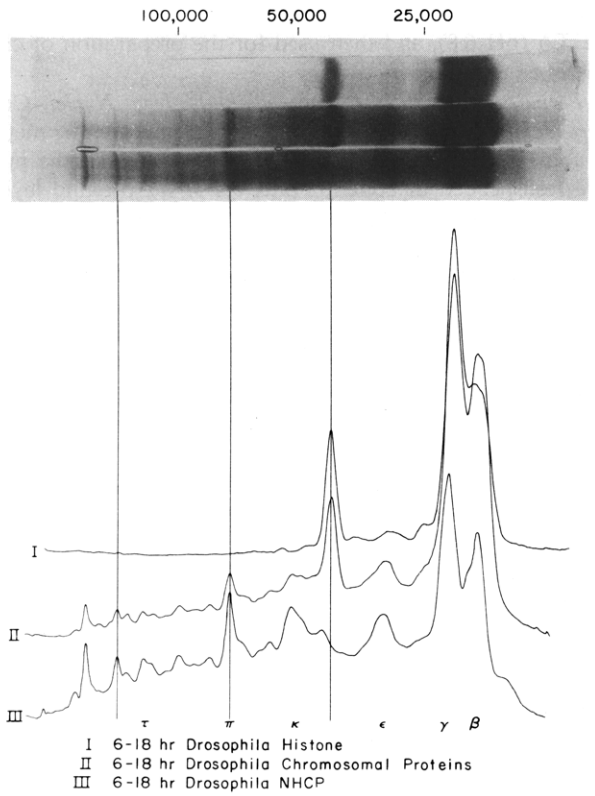


FIGURE 1: Sodium dodecyl sulfate–phosphate gel analysis of the chromosomal proteins of 6–18-hr *Drosophila* embryos.

tions were determined by the orcinol method (Dische and Schwarz, 1937) after hydrolysis in 0.3 M KOH; yeast RNA (Sigma) was used as a standard. Protein concentrations were determined by the method of Lowry *et al.* (1951) in 1 N NaOH after separation of the histones and NHC proteins by the method given above.

Template Activity. Template activities were assayed by incubation with *ca.* 0.5 μg of *Escherichia coli* F4 RNA polymerase prepared by the method of McConnell and Bonner (1972). The assay mixture (0.25 ml) contained 40 mM Tris (pH 8), 10 mM $MgCl_2$, 140 mM KCl, 0.8 mM potassium phosphate, 4 mM dithiothreitol, 11% glycerol, and 0.8 mM each of the nucleotide triphosphates. The label was [^{3}H]GTP, 20 μCi/mol. Following incubation at 37° for 10 min the reaction was stopped by addition of 1 ml of ice-cold 5 N NaCl and 4 ml of ice-cold 7% Cl_3CCOOH. After precipitating in ice for a minimum of 30 min, the samples were collected on Bac-T-Flex B-6 membrane filters (Schleicher and Schuell Co.). The filters were washed thoroughly with 7% Cl_3CCOOH, dried in a vacuum oven, and counted using a Beckman liquid scintillation system LS-200B in toluene scintillation fluid (6 g of 2,5-diphenyloxazole/l. of toluene).

Melting Curves. Samples were dialyzed extensively in the cold against 2.5×10^{-4} M EDTA (pH 8). Samples at a concentration of *ca.* 0.05 mg of DNA/ml were degassed in the cells and melted on a Gilford 2400 recording spectrophotometer at a rate of *ca.* 1°/2 min.

TABLE I: Chemical Composition of *Drosophila* Chromatin.

Source	DNA	RNA	Histone	NHC Protein
6–18-hr embryos	1	0.06	0.79	1.19
0–2-hr embryos	1	0.06	0.60	1.28

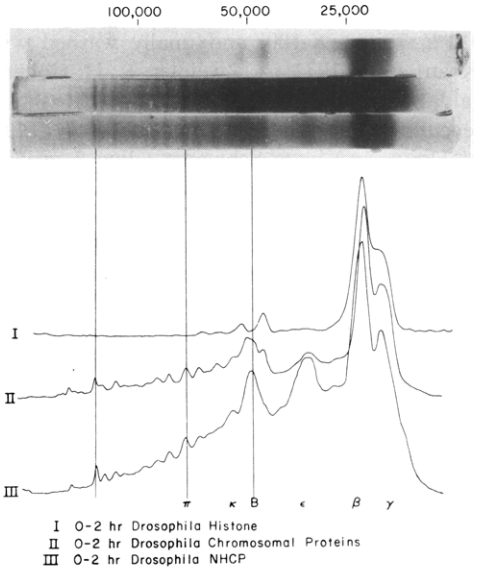


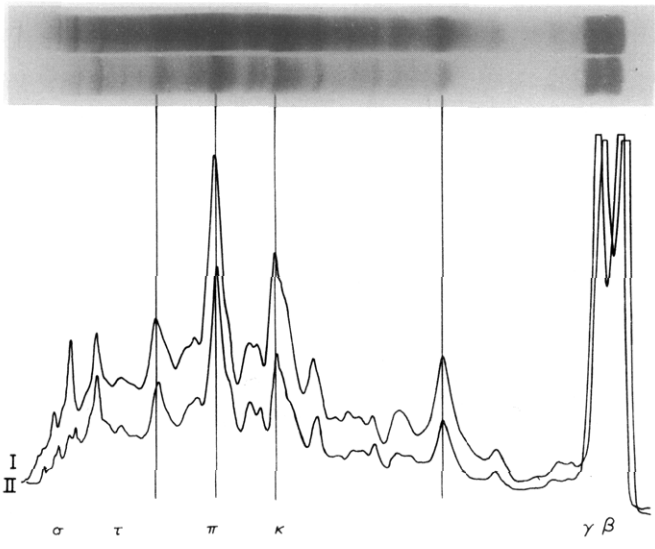
FIGURE 2: Sodium dodecyl sulfate–phosphate gel analysis of the chromosomal proteins of 0–2-hr *Drosophila* embryos.

Feulgen Staining of Drosophila Eggs. Egg samples were fixed and stained to assess the age distribution according to the method of W. J. Peacock and D. S. Hogness (personal communication). Eggs were dechlorinated, washed in saline, and fixed for 1 hr in 3:1:5 methanol–glacial acetic acid–toluene (two changes). They were then rehydrated by successive equilibration in 100, 95, 70, 50, 30, and 10% ethanol, and water, minimum 5 min in each. The last four solutions contain 0.1% Triton X-100. The DNA in the eggs was then hydrolyzed by their immersion in 1 N HCl plus 0.1% Triton X-100 at 60° for 8 min. Subsequently the eggs were washed with water and stained in Feulgen stain plus 0.1% Triton X-100 for 40–60 min. Following staining, early syncytial eggs will be white, blastula eggs will be pink, and cellular eggs will be deep purple, corresponding to the DNA content.

Materials. All chemicals were reagent grade unless otherwise specified. Polyacrylamide gel reagents were Electrophoresis Purity Reagents from Bio-Rad (Richmond, Calif.); sodium dodecyl sulfate (Sipon WD) was obtained from Alcolac Chemical Corporation (Baltimore, Md.). All urea used was purified by passage of a 10 M solution through a mixed bed ion exchange column (Barnstead DO 803).

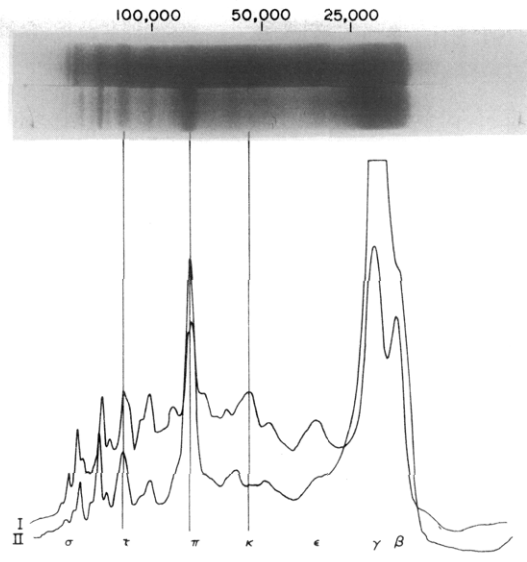
Results

Chromatin Preparation. Throughout this investigation attention will be focused on the preparations of chromatin from the blastula (0–2 hr) and post-gastrula (6–18 hr) embryonic stages of *Drosophila*. See the Discussion for justification. Chromatin can readily be prepared from all embryonic stages of *Drosophila* by the above methods. Assuming 900 μg of total DNA maximum/g of eggs (Phillips and Forrest, 1973), the yield of DNA as chromatin using 6–18-hr eggs and method 1 of preparation is 45–50%. The chromatin is typical in terms of its chemical composition (Table I), reflected also in the A_{230}/A_{260} of *ca.* 0.95 (Dingman and Sporn, 1964; Bonner *et al.*, 1968b). That the chromatin is not contaminated by other cellular or nuclear proteins is indicated by the absence of any significant very basic proteins other than histones (see Figures 1 and 5). The slowest moving band on the histone urea gels



I 6-18 hr Drosophila NHCP Regular Preparation
II 6-18 hr Drosophila NHCP Nuclear Preparation

FIGURE 3: Sodium dodecyl sulfate–Tris-glycine gel analysis of regular NHC protein preparation and that utilizing 2.2 M sucrose-purified nuclei.



I 6-18 hr Drosophila NHCP
II Tissue Culture Cell Drosophila NHCP

FIGURE 4: Comparison using sodium dodecyl sulfate–phosphate gels of NHC proteins of 6–18-hr *Drosophila* embryos and of *Drosophila* tissue culture cells.

has been shown to be the S–S dimer of histone III, in that the protein moves to the III position on the gels following reduction with β-mercaptoethanol.

Isolation of Histones and NHC Proteins. The results of the fractionation of chromatin into histones, DNA, and NHC proteins by the above methods are shown in Figures 1 and 2, sodium dodecyl sulfate–phosphate gel analysis (molecular weight sieving) of the total chromosomal proteins, histones, and NHC proteins.[2] A reasonably clean separation of the two protein classes has been achieved. Previous work has shown that the recovery of total protein in this type of procedure is very good (90% or better) (Elgin and Bonner, 1970) and that the reproducibility of the gel patterns is excellent (Wu *et al.*, 1973). Reassuringly, the sum of the two protein fraction gel patterns gives the total chromosomal protein gel pattern, indicating that no artifacts are generated during the fractionation procedure. (The total chromosomal protein sample was obtained simply by dialyzing a chromatin aliquot against the desired running buffer.)

The NaCl–HCl histone extraction procedure used here, while the best of several methods tried, is not completely satisfactory. Further extraction of the resulting pellet with 0.25 N HCl yields an additional 10% histone (indicating that the initial extraction procedure removed 90% of the histone). Unfortunately, this second extract contains an equal amount of NHC protein as shown by disc gel analysis, and so was not routinely used. The residual 10% histone in the NHC protein fraction is predominantly histones II, III, and IV; thus the NHC protein bands β and γ are contaminated with these histones. That bona fide NHC proteins are also present at positions β and γ is indicated by the observations that (1) significant protein persists at these molecular weight positions (particularly γ) even after exhaustive acid extraction of *Drosophila* chromatin (gels not shown) and (2) bands at this position in rat liver NHC protein preparations have been shown to be acidic proteins by amino acid analysis (Elgin and Bonner, 1970, 1972).

Chemical Composition of Drosophila Chromatins. The 0–2- and 6–18-hr embryo chromatins were analyzed for composition by the methods described. The results are given in Table I in terms of mass ratios relative to DNA. The protein figures have been corrected for the histone extraction problem discussed above. Standard deviations are *ca.* 15%. The data are consistent with the melting curve analysis of these chromatins (see below).

Control: Preparations of Chromatin from Purified Nuclei. The procedure routinely used for preparation of chromatin, method 1, entails isolation of a crude nuclear pellet only. To ensure that no cytoplasmic contamination resulted from this practice, chromatin was prepared from 2.2 M sucrose purified nuclei, the proteins extracted, and gel patterns compared. No variance was observed in the histone pattern. As can be seen in Figure 3, the NHC protein gel pattern from this preparation is identical with the routine preparation over most of the molecular weight range. An observable difference occurs at very high molecular weights (top of gel); this involves less than 3% of the total protein. Analogous results were obtained in the case of 0–2-hr embryo chromatin (gels not shown).

Chromosomal Proteins of Drosophila Tissue Culture Cells. Chromatin was prepared from *Drosophila* tissue culture cells (Schneider's line 2) as given above, the chromosomal proteins extracted, and the gel patterns compared with those from 6–18-hr embryo chromatin. No differences were observed in the histone pattern on urea gels. Two differences were observed in the NHC protein gel pattern (see Figure 4). The tissue culture cell NHC proteins were deficient in the amount of κ, although a faint band can definitely be observed at this position. In the very high molecular weight region (σ), the band pattern differed from that of the regular 6–18-hr embryo NHC protein preparation, more nearly resembling that of the 6–18-hr embryo NHC protein preparation made from sucrose-purified nuclei. This suggests that the quantitatively reduced high molecular weight band could be a nuclear membrane protein. In general, however, the two NHC protein

[2] For convenience in discussion, the NHC protein bands have been given arbitrary Greek labels (see Figure 6). These are roughly homologous, in terms of molecular weight, to those used for rat liver (Elgin and Bonner, 1970; Wu *et al.*, 1973).

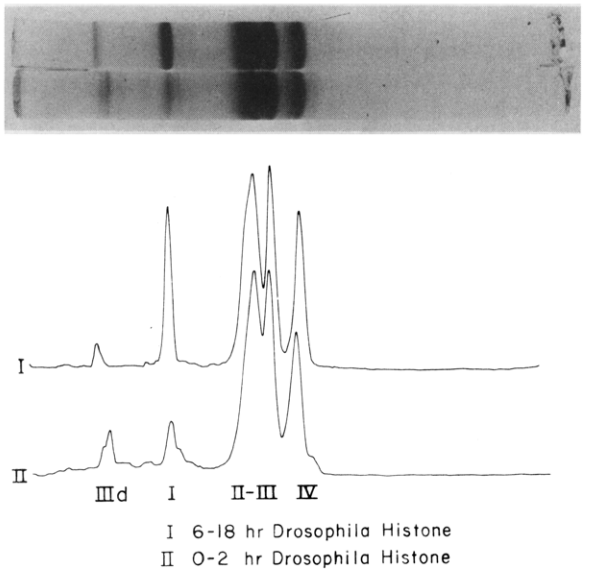


FIGURE 5: Comparison using urea gels at pH 4.3 of blastula and 6–18-hr embryo *Drosophila* histones.

band patterns are quite similar and represent a good NHC protein base line for general embryonic *Drosophila*.

Changes in Chromosomal Proteins during Embryonic Development. Inasmuch as there is considerable change in the activity of the chromatin, both in terms of DNA polymerase and of RNA polymerase, during the embryonic development of *Drosophila* it is of interest to look for changes in the chromosomal proteins over this period. Figure 5 presents a comparison of the histones from 0–2-hr embryos (blastula and preblastula) and from 6–18-hr embryos. The salient feature is the decrease in histone I in the blastula pattern. It should be noted that histone I is the histone most susceptible to artifacts of extraction and protein degradation. While no definitive work on histone protease in *Drosophila* is yet available, preliminary experiments indicate little or no degradation of histones on incubation of 6–18-hr embryo chromatin at 37°, pH 8, for several hours.

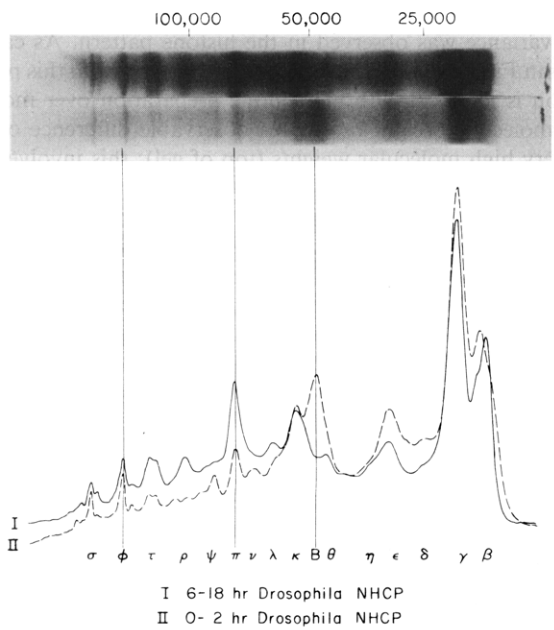


FIGURE 6: Comparison using sodium dodecyl sulfate–phosphate gels of blastula and 6–18-hr embryo *Drosophila* NHC proteins.

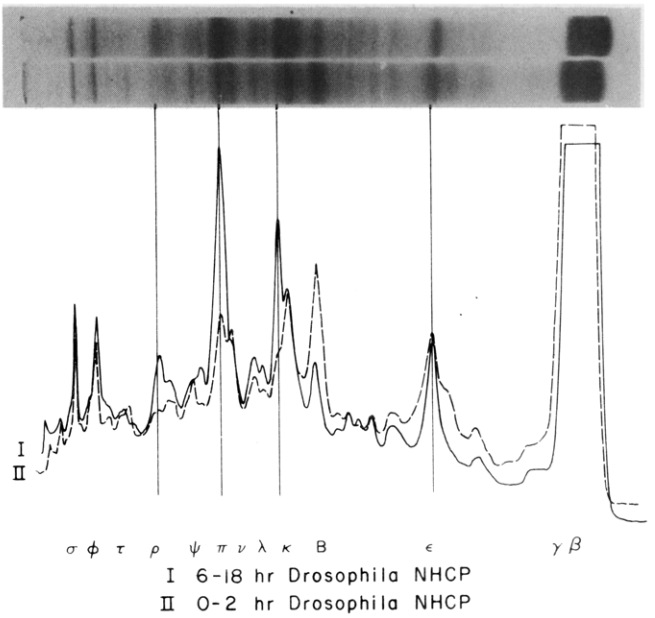


FIGURE 7: Comparison using sodium dodecyl sulfate–Tris-glycine gels of blastula and 6–18-hr embryo *Drosophila* NHC proteins.

Two striking differences are noted in a comparison of the 0–2- and 6–18-hr embryo NHC proteins on sodium dodecyl sulfate–phosphate gels (Figure 6). The 0–2-hr embryo NHC protein contains a prominent band of mol wt ~45,000 (band B) which is not apparent in the 6–18-hr embryo NHC protein. Further, there is a difference in the protein distribution among three bands in the mol wt 100,000 region of the gel (bands ρ). Other quantitative differences may be noted. Analysis of the two NHC protein patterns on sodium dodecyl sulfate–Tris-glycine gels suggests that the difference at position B, like the others, may be more quantitative than qualitative (Figure 7); however, a clear difference in the nature of the bands is still apparent. Except as noted, considerable similarity exists between the band patterns. A study of the NHC proteins of *Drosophila* embryos collected at 4-hr intervals indicates a smooth transition from the blastula pattern to the older embryo pattern for both NHC proteins and histones (Figures 8 and 9). For the NHC proteins the bulk of the transition has occurred at 6 hr and it is complete at 10 hr. Histone I increases apparently between 2 and 10 hr and then stabilizes at a constant level.

It is important to know the accuracy of the timing in the collection of embryos, particularly for the early collections. This can be estimated by staining eggs from a 2-hr collection (0–2-hr embryos) with Feulgen stain by the method given above; 2580 eggs were stained and counted: 2306 or 89.4% were white (preblastula), 255 or 9.9% were pink (blastula), and 19 or 0.7% were purple (cellular). Assuming that the blastula eggs have *ca.* 2×10^3 nuclei each (Sonnenblick, 1950) and that the cellular eggs have at a maximum a full complement of 4.5×10^5 nuclei (Phillips and Forrest, 1973), this will result in a chromatin that is maximally 50% from older embryos, the remainder being from preblastula and blastula embryos. Thus, the effects noted are probably underestimates of the true differences between blastula and older embryo chromatin.

Comparative Characterization of Blastula Chromatin. To assist in evaluating the significance of the above observations, the 0–2-hr embryo chromatin (blastula) was characterized as to melting behavior and template activity relative to 6–18-hr

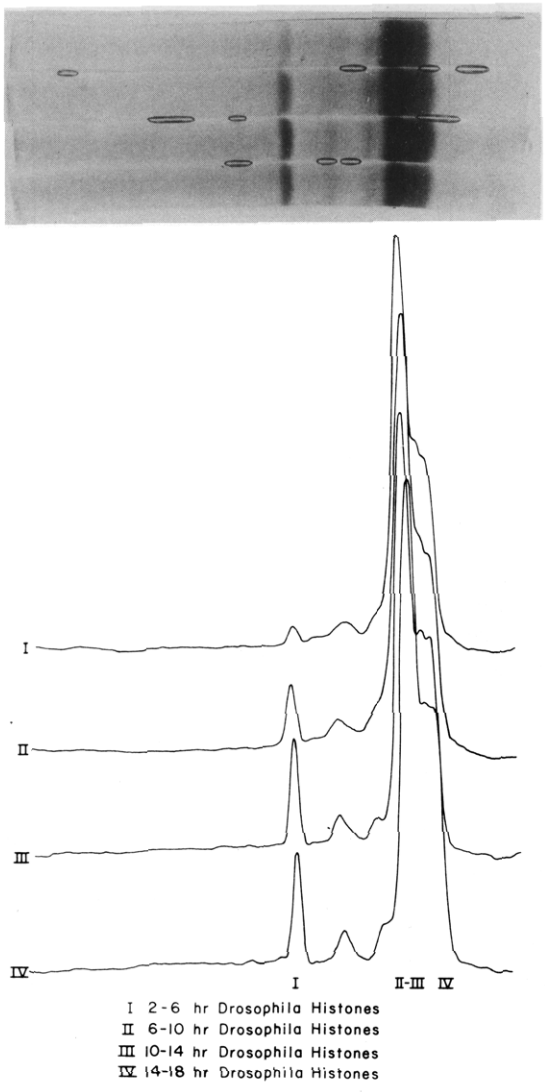


FIGURE 8: Time course of change in *Drosophila* embryo histone pattern, sodium dodecyl sulfate–phosphate gels.

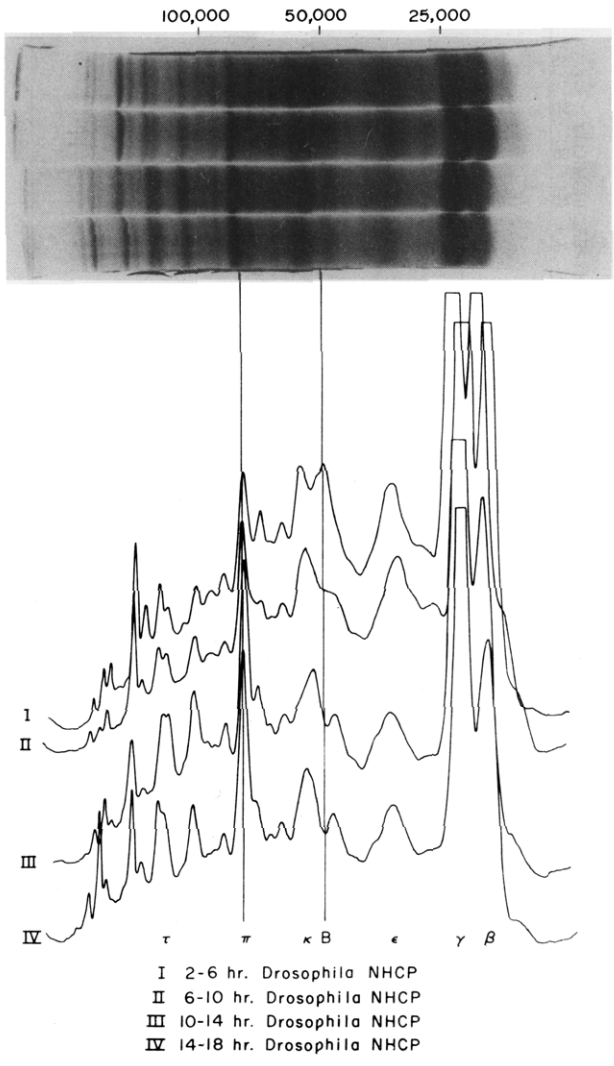


FIGURE 9: Time course of change in *Drosophila* embryo NHC protein pattern, sodium dodecyl sulfate–phosphate gels.

embryo chromatin. The melting curves, determined as described under Methods, are presented in Figure 10. *Drosophila* DNA was melted concurrently, and these data are included as a reference and control. It has previously been shown that under these conditions the proportion of the DNA melting in the temperature range 65–95° will be directly related to the histone:DNA ratio and the final hyperchromicity will be inversely related to the histone:DNA ratio (Shih and Bonner, 1970; Smart, 1970; Smart and Bonner, 1971a). As may be predicted on the basis of the chemical composition data (Table I), the 0–2-hr embryo chromatin shows a higher percentage of low-temperature melting material, a lower percentage of high-temperature melting material, and a slightly greater hyperchromicity. The numerical parameters of the data are given in Table II.

The template activity *in vitro* of the chromatins relative to that of *Drosophila* DNA was assessed using *E. coli* RNA polymerase in a standard reaction mixture. The resulting data are presented in Figure 11. At saturation, the relative template activity of 6–18-hr embryo chromatin is 16.4%, while that of 0–2-hr embryo chromatin is 34.9%, or 2.2-fold greater. The endogenous activity of both chromatins is *ca.* 3–4%.

Discussion

One purpose of this work has been to establish routine methods for the preparation of *Drosophila* embryo chromatin in good yield and to demonstrate that such chromatin is typical of that of eukaryotes in chemical and biological properties. The use of *Drosophila* embryos rather than older developmental stages increases the efficiency in production of starting material and minimizes the hazard of yeast contamination and of the presence of cellular nucleases and proteases. It can be estimated that, gram for gram, *Drosophila* 6–18-hr embryos are eight times as costly as rat liver, but only a little less convenient to use in such experiments. Previous data in the literature on *Drosophila* histones are in agreement with

TABLE II: Melting Characteristics of *Drosophila* Chromatin.

Sample	T_m (°C)	Hyperchromicity (%)
DNA	43.0	31.3
0–2-hr embryo chromatin	65.4	28.0
6–18-hr embryo chromatin	71.0	27.4

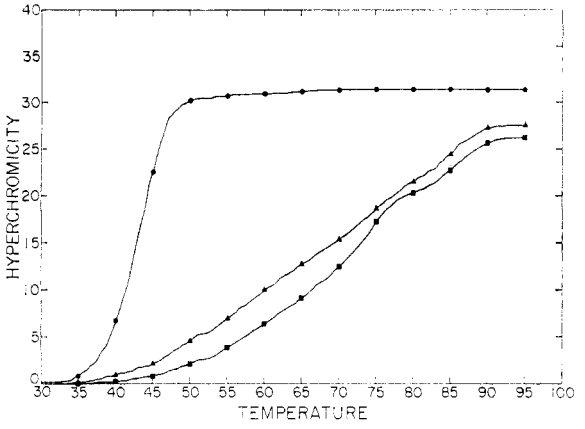


FIGURE 10: Melting curves of *Drosophila* DNA (●), 0–2-hr embryo chromatin (▲), and 6–18-hr embryo chromatin (■) in 2.5×10^{-4} M Na_2EDTA, pH 8.0.

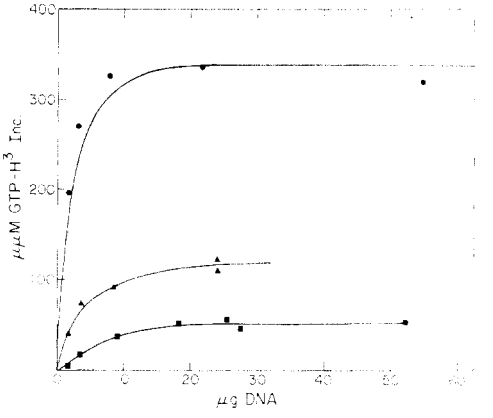


FIGURE 11: Template activity of *Drosophila* DNA (●), 0–2-hr embryo chromatin (▲), and 6–18-hr embryo chromatin (■) using *E. coli* F4 RNA polymerase.

that obtained here (Panyim *et al.*, 1970; Cohen and Gotchel, 1971; Oliver and Chalkley, 1972), as are certain characteristics of the general embryo chromatin (S. F. Harris and J. Bonner, personal communication). The base-line parameters now seem well established.

The second purpose of these experiments has been to explore possible differences in the chromosomal proteins during development. The blastula of *Drosophila* is unusual in being a syncytial egg (no cell walls within the egg) and in having very rapid nuclear division. During the first 2 hr after fertilization the nuclei complete a replication cycle once every 10 min. At 24°, it is estimated that interphase requires 3.4 min, prophase requires 4.0 min, metaphase 0.3 min, anaphase 1.0 min, and telophase 0.9 min (Sonnenblick, 1950). At the end of this period the nuclei migrate to the periphery of the egg, and cell walls begin to form between them. It is at this point that nucleoli first appear (*ca.* 2.5 hr) (Sonnenblick, 1950), and work in other systems suggests that heterochromatization (of the appropriate regions) and greatly increased transcription also occur at this time (Fristrom, 1970; Chen, 1971). At the end of the third hour the cell walls have finished forming around the nuclei and the egg is the typical ball of cells surrounding the yolk. As development proceeds, the average rate of cell division (and nuclear division) is *ca.* once per hour, until between hours 10 and 11 mitotic activity ceases in all parts of the embryo except in the nervous system (Poulson, 1950). The total cell number is 4.5×10^5 cells/egg (Phillips and Forrest, 1973).

Such extreme changes in the replication rate and state of the chromatin should lead to visible differences in the chromosomal protein pattern if these are correlated; such differences are here reported. *A priori*, the differences in the protein patterns might be related to (1) the rate of nuclear division, (2) the changes in chromosomal activities noted above at 2–3 hr, or (3) some other factor not yet delineated. Because of the gradual nature of the shift in the chromosomal protein gel patterns (2–6-hr embryo NHC protein patterns definitely contain elements of the 0–2-hr pattern), the first seems more likely than the second. Serious contamination of older stages by younger stages is very unlikely due to the geometric progression in the nuclear age distribution.

The rate of division of the syncytial nuclei has two consequences. First the chromatin is *ca.* two-thirds in a condensed state (time average, prophase through telophase). Second, the concentration of replication forks is unusually high, an estimated 1×10^4 forks per nucleus (time averaged) (Blumenthal *et al.*, 1973). It will be of interest to determine whether the "condensed" or "replicative" chromatin states can be specifically correlated with the unusual properties of blastula chromatin reported here, *i.e.*, its high template activity and altered chromosomal protein pattern.

Previous studies have determined that the metaphase condensed form of chromatin has relatively low template activity (Fan and Penman, 1970; Farber *et al.*, 1972). In contrast, it might be anticipated that the large number of nicks and breaks which the replication forks constitute could act as initiating points for *E. coli* RNA polymerase, resulting in the high template activity observed. However, the shearing of chromatin (as is routinely done here) has been found to increase the template activity of metaphase preparations (McClure and Hnilica, 1973; J. Bonner, personal communication).

Little data are as yet available on the NHC proteins characteristic of different states of chromatin. Two studies comparing the NHC proteins of mammalian metaphase chromosomes and interphase chromatin of the same tissue indicate that the proteins are quite similar (Elgin *et al.*, 1973; Comings and Tack, 1973). In particular, no dominant new protein, such as the B protein, is observed. One might then speculate that the B protein is characteristic of the replicative form of chromatin. The only protein known to be required in large amounts for DNA replication is the T4 gene 32 protein described by Alberts and Frey (1970); calculations indicate that the B protein is present in an appropriate amount for such a stoichiometric role (Elgin *et al.*, 1973). Further study of this possibility is in progress.

This work is in agreement with several other studies indicating that normal histones are found associated with the genome in very early embryos. Juvenile histones, which have been reported for *Drosophila* on the basis of cytological observations (Das *et al.*, 1964), must be confined to the very early stages (first few rounds of nuclear division) if they exist at all. In other organisms normal histones are observed at blastula, and even as early as the first cleavage (sea urchin, Marushige and Ozaki, 1967, and J. V. Rudderman and P. R. Gross, personal communication; *Xenopus*, Byrd and Kasinsky, 1973). Several investigations have indicated that early embryos are deficient in histone I, as reported here (pea bud cotyledon, Fambrough *et al.*, 1968; sea urchin, Orengo and Hnilica, 1970). Whether or not this is a consistent artifact of degradation is unknown; however, *in vitro* translation of sea urchin embryo histone mRNA results in synthesis of histones

with a deficiency of I (Gross *et al.*, 1973). This suggests that the observation is valid. The only property of chromatin known to be uniquely related to histone I is its solubility under various salt conditions (Smart and Bonner, 1971a,b). The functional significance of these observations is unknown.

Drastic changes in the NHC protein pattern during development of the sort reported here (Figure 6) have not been found previously, although quantitative changes have been observed in studies of sea urchin NHC proteins (Hill *et al.*, 1972; Cognetti *et al.*, 1972). It may be that this is a consequence of the fact that in no other system does nuclear division occur so rapidly, and thus in no other system can one obtain the contrast of states observed here. The high concentration of replication forks found in the syncytial egg surpasses even what is available in synchronized cells, and this fact recommends *Drosophila* as a system to those interested in studying chromosome replication in eukaryotes.

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