Extracts of *Drosophila* embryos mediate chromatin assembly *in vitro*

(nucleosome core particle/histones/supercoiling/micrococcal nuclease)

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ABSTRACT   Extracts of *Drosophila* embryos can mediate the assembly of a chromatinlike structure from histones and DNA under physiological conditions. The histone–DNA complex formed *in vitro* contains micrococcal nuclease-sensitive sites spaced at 200-base pair intervals. More extensive digestion of the complex by micrococcal nuclease generates 11S particles which cosediment with nucleosome core particles isolated from native chromatin. These particles contain 140-base pair DNA fragments which upon further cleavage with micrococcal nuclease give rise to a pattern of discretely sized DNA fragments characteristic of nucleosome core particles. We have assayed the chromatin assembly process both qualitatively by measuring the induction of supertwists into a relaxed circular DNA (a process requiring a nicking–closing enzyme) and quantitatively by measuring the formation of micrococcal nuclease-resistant DNA fragments from radioactively labeled linear DNA. The amount of chromatin formed depends primarily on the amount of histones, whereas the rate of assembly depends on the amount of extract protein added. The factors in the extract that mediate chromatin assembly appear to interact first with the DNA because preincubation of the DNA with the extract markedly increases the extent of assembly.

The ability of histones and DNA to associate to form a nucleosomelike structure upon dialysis from concentrated salt solutions to physiological ionic strengths indicates that these components alone contain much of the information essential for forming native chromatin. Reconstitution of chromatin from histone and DNA has been a very useful method for identifying the components required for nucleosome formation (1). Chromatin reconstituted by dialysis of histone/DNA mixtures from high ionic strengths also fulfills many of the other criteria used to judge a native structure—i.e., a repeating pattern of nuclease sensitive sites (2), the formation of nucleosome core particles (3), and the supertwisting of DNA (4). Although reconstitution of chromatin allows an analysis of the structural requirements for chromatin, the nonphysiological ionic conditions used during reconstitution are inconsistent with studies of the cellular mechanism of chromatin assembly.

The physiological mechanism of chromatin assembly has been examined primarily *in vivo* (5–8). On the other hand, an *in vitro* system is necessary for the fractionation and identification of the functional components of chromatin assembly. Laskey *et al.* (9) have shown that extracts of *Xenopus* eggs can assemble histones and simian virus 40 DNA into minichromosomes *in vitro*. Moreover, they were able to purify a protein from these extracts that associates with histones and then transfers the histones to DNA to form a chromatinlike structure (10).

We have studied the assembly of chromatin in extracts of *Drosophila* embryos because of their rapid rate of DNA synthesis and presumably chromatin assembly (11). We show that an extract prepared from these embryos can convert free histones and DNA into a complex similar to native chromatin by

several physical criteria. Assembly appears to occur in more than one step, with the extract interacting with the DNA before the DNA interacts with histones.

MATERIALS AND METHODS

Preparation of Extract. Embryos of *Drosophila melanogaster* (Oregon-R) of average age 1 hr were collected on feeding plates, washed with saline solution (0.7% NaCl/0.1% Triton X-100) at 0°C, dechorionated in 1.25% sodium hypochlorite for 90 sec, and washed again in saline. After settling twice from saline and once from homogenization buffer (0.25 M sucrose/20 mM Tris·HCl/1 mM CaCl₂/2 mM EDTA/100 mM NaCl at pH 7.5) to remove debris, the embryos (10 g) were homogenized in 40 ml of homogenization buffer with three strokes of the B pestle in a Dounce homogenizer. Nuclei were *removed* by sedimentation at 2000 × *g* for 10 min, and the supernatant was further fractionated by centrifugation at 15,000 × *g* for 20 min. Aliquots of the final supernatant were stored at −20°C and could be frozen and thawed 5–10 times without measurable loss of activity.

Preparation of Assembly Substrates. *Escherichia coli* DNA was labeled with [³H]thymidine (40 Ci/mmol; 1 Ci = 3.7 × 10¹⁰ becquerels) *in vivo* and extracted according to Katz *et al.* (12). Pellets containing bacterial DNA from the detergent-lysed cells were extracted four times with phenol that had been equilibrated with TE buffer (10 mM Tris·HCl/1 mM EDTA at pH 7.4) and once with chloroform/isoamyl alcohol (24:1). The DNA was then precipitated with ethanol and resuspended at about 1 mg/ml in TE buffer. The DNA was treated with RNase A at 20 μg/ml for 20 min at 37°C, extracted with phenol, chromatographed on A15m agarose, and reprecipitated with ethanol. DNA stock solutions (specific activity of 6000 cpm/μg and of broad chain length between 5 and 20 kilobases) were adjusted to 100 μg/ml after a brief sonication and were stored at −20°C. Unlabeled salmon sperm DNA (Worthington) was phenol extracted. Plasmid pBR322 DNA and bacteriophage PM2 DNA were isolated as described (12, 13).

Chromatin was isolated from *Drosophila* tissue culture cells [diploid Kc cell line (14)] by the method of Hancock *et al.* (15). A mixture of the four core histones was prepared from this chromatin by first extracting histone H1 with 0.6 M NaCl for 30 min and pelleting the chromatin by centrifugation for 40 hr at 320,000 × *g* at 4°C. The core histones were then acid extracted according to Germond *et al.* (16). Sodium bisulfite (1 mM) was present at each preparative step to inhibit proteolysis (17). Histones were stored at −20°C at a concentration of 2–4 mg/ml in 1 mM HCl/5 mM dithiothreitol. The histones used in these experiments were intact and not detectably modified as judged by gel electrophoresis on long (30 cm) acid/urea/polyacrylamide slab gels (18, 19).

Chromatin Assembly Assay. *E. coli* [³H]DNA (1 μg) or plasmid [³H]DNA was incubated in homogenization buffer with 3 μg of extract protein and 1 μg of core histones (added last) at 30°C unless stated otherwise. The reaction was quenched by the addition of a 50-fold excess of unlabeled *E. coli* or salmon

sperm DNA, made 5 mM in CaCl₂, and treated with 30 units of microccocal nuclease for 30 min at 37°C. The digestion was stopped and acid-precipitable counts were measured by addition of EDTA (10 mM), undigested carrier DNA (25 μg), and trichloroacetic acid (to 3%) and collection on Whatman GF/C filters that had been wetted in wash solution (1 M HCl/0.1 M sodium pyrophosphate). The filters were then washed four times with 5 ml of ice cold wash solution and once with 5 ml of ethanol and dried before counting. Time courses were performed by removing aliquots containing 1 μg of DNA from a large-scale reaction and then treating the aliquots as described above.

Preparation of Native and Assembled Core Particles. Core particles were prepared by treating *Drosophila* tissue culture chromatin with microccocal nuclease and purifying the digestion products on sucrose gradients as described (20). Core particles from chromatin assembled *in vitro* were prepared by treating a large-scale assembly reaction containing 2.5 μg of [³H]DNA (25,000 cpm total), 2.5 μg of histone, and 10 μg of embryo extract with microccocal nuclease as above. Approximately 80% of the nuclease-resistant product was recovered on the sucrose gradient as measured by acid precipitation of aliquots from each fraction.

Microccocal Nuclease Digestion of Native and *In Vitro* Assembled Core Particles. Core particles prepared as described above were adjusted to 20 μg/ml and made 2 mM in CaCl₂. Microccocal nuclease was added to 160 units/ml and the reaction mixture was incubated for 1.5 hr at 37°C. The digestion was terminated with EDTA (10 mM) and sodium dodecyl sulfate (0.1%). The DNA fragments were prepared for electrophoresis by treatment with proteinase K (200 μg/ml) for 2 hr at 37°C and by ethanol precipitation.

RESULTS

Extracts of Drosophila Embryos Convert DNA into a Chromatinlike Structure. We first detected chromatin assembly activity in extracts of *Drosophila* embryos by using the supercoiling assay described by Laskey *et al.* (9). This assay is based on the observation of Germond *et al.* (4) that DNA becomes supercoiled by one helical turn as it associates with each histone octamer. Germond *et al.* (4) showed that when a relaxed circular DNA and histones are reconstituted by dialysis from high ionic strengths to form nucleosomes, the resulting complex is in a compact, twisted conformation. After relaxing such complexes with a nicking–closing enzyme and then isolating the DNA free of histones they found that it had become supercoiled in proportion to the number of nucleosomes formed. This assay for chromatin assembly depends on both the interaction of histones with DNA and the activity of a nicking–closing enzyme to relax the histone–DNA complex.

When relaxed, covalently closed circular pBR322 DNA and an equal weight of the four core histones were incubated with an extract from *Drosophila* embryos at physiological ionic strengths, the DNA became supertwisted (Fig. 1). These DNA molecules were supertwisted in the negative sense because they could be relaxed by the addition of ethidium bromide (data not shown). The extent of supercoiling produced by a fixed amount of extract depends on the amounts of both histones and DNA added to the reaction. The extent of supercoiling was maximal at a histone-to-DNA ratio of 2:1 by weight (data not shown), although a 1:1 ratio was used for most of the experiments described below. In the absence of exogenous histones, 400 μg of extract protein is capable of supertwisting 0.01–0.02 μg of PM2 DNA, presumably as a result of a pool of endogenous histones. The same amount of extract will induce supertwists in 10 μg of PM2 DNA if an equal weight of the four core histones is present. The presence of DNA molecules in some of these re-
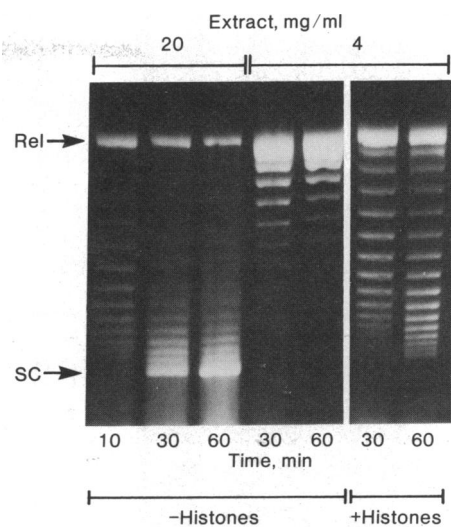

FIG. 1. Incubation of relaxed circular DNA in extracts of *Drosophila* embryos results in induction of superhelical turns. Relaxed and covalently closed pBR322 DNA (0.15 μg) was incubated with either 4 or 20 mg of embryo extract per ml in a 20-μl reaction mixture. Core histones (0.15 μg) were also included in the two reactions shown on the right. Reactions were terminated at the indicated times and the mixtures were deproteinized prior to electrophoresis in a 1% agarose slab gel in 0.09 M Tris/0.09 M boric acid/2 mM EDTA at pH 8.3 (TBE buffer) at 3 V/cm. The gel was stained for 10 min in 0.5 μg of ethidium bromide per ml, destained for 5 min, and photographed through an orange filter while illuminated by a short-wavelength UV lamp. The relaxed pBR322 substrate DNA (Rel) and a physiologically supertwisted pBR322 DNA (SC) migrated as indicated.

actions intermediate between relaxed and fully supercoiled forms indicates incomplete assembly and suggests that the association of histones and DNA may not be cooperative.

Chromatin Assembled *In Vitro* Contains Microccocal Nuclease-Sensitive Sites at Regular Intervals. Limited digestion of the product of the *in vitro* assembly system with microccocal nuclease revealed a pattern of sensitive and resistant regions similar to that present in native chromatin (Fig. 2). Sensitive sites occurred at 200-base pair intervals when chromatin was assembled with large amounts of extract and no exogenous histones. More extensive digestion of the chromatin assembled *in vitro* resulted in the production of discretely sized DNA fragments 140 base pairs in length. The 200-base pair pattern produced by limited microccocal nuclease digestion of native chromatin results from the regular spacing of nucleosomes along the DNA, whereas the 140-base pair fragment corresponds to that DNA specifically protected from digestion by the octamer of the core histones (H2A₂H2B₂H3₂H4₂; for review see ref. 21). Microccocal nuclease digestion of the DNA–histone complex assembled in the presence of limiting amounts of extract and exogenous core histones revealed a more disperse but still regular repeating pattern of sensitive regions and the same 140-base pair resistant DNA fragment (data not shown). This increased heterogeneity could reflect a stoichiometric requirement for some other protein in the extract such as histone H1 or some modified form of the core histones for the correct spacing of the nucleosomes along the DNA. In no case were discretely sized DNA fragments generated from DNA–histone complexes formed in the absence of the embryo extract.

Chromatin Assembled *In Vitro* Contains 11S Core Particles. The organization of chromatin assembled *in vitro* was compared to native chromatin isolated from *Drosophila* tissue culture cells. Microccocal nuclease digestion of both chromatins under conditions that degraded the DNA to 140-base pair fragments led to nucleoprotein particles that sedimented at 11S
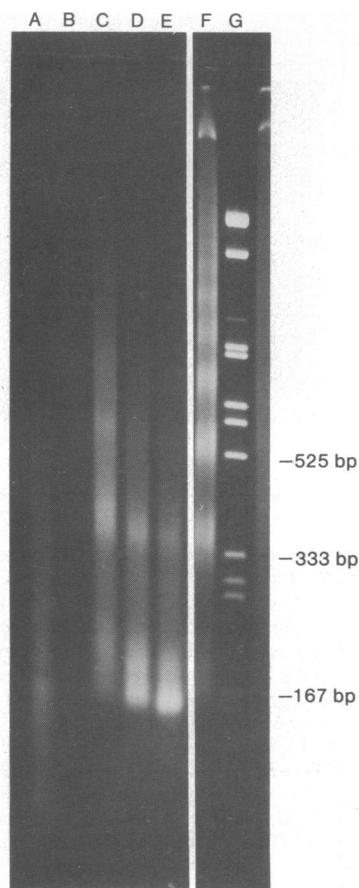
FIG. 2. Chromatin assembled *in vitro* contains micrococcal nuclease-sensitive sites at regular intervals. Relaxed circular PM2 DNA (1 μg) was incubated in 20 μl of embryo extract at 20 mg/ml (utilizing endogenous histones) for 60 min at 30°C. CaCl₂ (2 mM) was added and the mixture was treated with 80 units of micrococcal nuclease per ml for 3 min (lane C), 9 min (lane D), or 18 min (lane E) at 37°C, RNase treated, deproteinized, and electrophoresed as described in Fig. 1. Control incubations were performed as above but without extract (lane A) or without added DNA (lane B). The markers include the pattern of fragments derived from micrococcal nuclease digestion of native chromatin (lane F) and *Hae* III-digested PM2 DNA (lane G). bp, Base pairs.

in isokinetic sucrose gradients (20, 22). Fig. 3 shows that the nucleoprotein particles from chromatin assembled *in vitro* cosediment with nucleosome core particles from native chromatin and that the assembly *in vitro* depends on the presence of the embryo extract.

When 11S particles were purified from chromatin assembled *in vitro* and then digested further with micrococcal nuclease, a pattern of subnucleosomal DNA fragments was produced which was qualitatively the same as the pattern of fragments generated from native core particles (Fig. 4). The lengths of the DNA fragments in the two patterns were identical. The difference in amounts of each fragment represents different extents of digestion, which is often seen with native core particles.

Assembly of DNA into Chromatin Can Be Measured Quantitatively by Its Resistance to Micrococcal Nuclease.

When chromatin was assembled *in vitro* from ³H-labeled circular DNA and an equal weight of the core histones, the 140-base pair DNA fragments resulting from digestion with micrococcal nuclease could be measured accurately by acid precipitation (Fig. 5). The rate of appearance of acid-precipitable, micrococcal nuclease-resistant fragments was parallel to the induction of negative supertwists, but acid precipitation gave
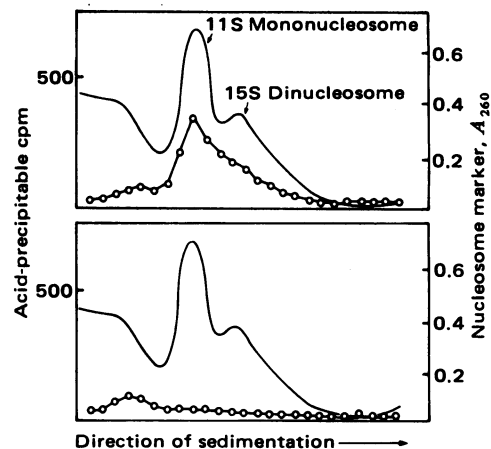

FIG. 3. Core particles from native chromatin and chromatin assembled *in vitro* cosediment. Native and assembled core particles were prepared, sedimented, and fractionated. (*Upper*) Sedimentation profiles of *in vitro* assembled core particles from the complete reaction (O—O) and of native core particles (——). (*Lower*) Profiles of an assembly reaction in which the extract was not present (O—O) and of the control core particles (——). Sedimentation patterns identical to *Lower* were obtained from complete assembly reactions that had been incubated at 0°C rather than at 30°C.

a quantitative measure of the number of nucleosomes assembled *in vitro*. Because this assay depends on the formation of the 140-base pair size limit in micrococcal nuclease digestion, it is rather insensitive to the precise time of nuclease digestion. This assay allows measurement of assembly by using labeled linear DNA rather than circular forms. The histone–DNA complexes formed in the absence of the extract were sensitive to nuclease digestion with no more than 2–5% being acid precipitable.

One microgram of core histones could protect up to 500 ng of DNA (see below and Fig. 4). The extent of reactions containing limiting amounts of histones could be increased by the
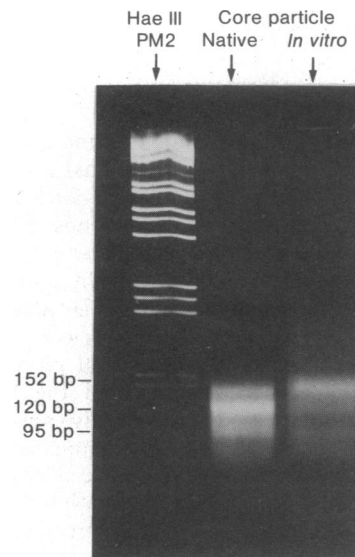

FIG. 4. Extensive micrococcal nuclease digestion of isolated core particles gives rise to discrete subnucleosomal DNA fragments. *In vitro* assembled and native core particles were isolated on sucrose gradients as in Fig. 3 and were further digested with micrococcal nuclease. DNA was deproteinized and electrophoresed in a 5% acrylamide slab gel in TBE buffer at 10 V/cm for 3 hr. DNA size standards were prepared by digesting PM2 DNA with *Hae* III restriction endonuclease. bp, Base pairs.
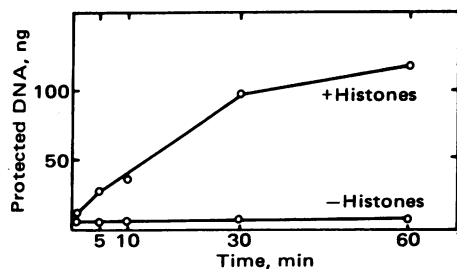
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FIG. 5. Kinetics of chromatin assembly reaction. Five micrograms of [^{3}H]pBR322 DNA (6000 cpm/μg) and 5 μg of embryo extract were incubated in the assembly reaction for varying times at 30°C in the presence or absence of an equal weight of core histones. Acid-precipitable counts were measured after micrococcal nuclease digestion of aliquots (0.5 μg). The induction of superhelical turns was measured in separate aliquots of this same reaction and their appearance paralleled the formation of nuclease-resistant, acid-precipitable counts (data not shown).

addition of more histones even after incubation for 60 min. This indicates that depletion of histones, and not inactivation of the assembly activity, is responsible for the limited reaction. We have avoided the use of histones in excess of the weight of DNA because such high levels can cause both precipitation during the reaction and elevated backgrounds of micrococcal nuclease-resistant DNA fragments (>10% of the total DNA). The histones could not be replaced by either polyamines or various other basic proteins. All other proteins tested protected less than 1% of the DNA under our assay conditions (Table 1).

Rate of Chromatin Assembly Depends on Amount of Extract. We used the DNA protection assay described above to measure the initial rates of chromatin assembly. Kinetics were determined as shown in Fig. 5 except that *E. coli* [^{3}H]DNA was used as substrate. Fig. 6 shows that the initial rate of assembly reaches a maximum of 4 ng of chromatin assembled per min in the presence of 6 μg of extract protein under our standard assay conditions. Larger amounts of extract protein produced only a small increase in reaction rate. Even at the highest level of extract used in these experiments (90 μg of protein per assay), the protection of DNA was dependent upon the addition of exogenous histones. If the embryo extract was pretreated with trypsin (0.02 μg of trypsin per μg of extract protein at 37°C for 20 min) or *N*-ethylmaleimide (10 mM for 30 min) or heated (60°C for 5 min) the activity was rapidly lost (data not shown).

Preincubation of DNA with Extract Increases Extent of Chromatin Assembly. When DNA was preincubated with the embryo extract prior to the addition of core histones, the rate of assembly was stimulated and the extent was increased (Fig.

Table 1.   Histones are required for formation of micrococcal nuclease-resistant DNA

Histone or replacement	DNA protected, ng/60 min
Core histones (33 μg/ml)	143
Spermidine (2 mM)	13
Spermine (2 mM)	8
Putrescine (5 mM)	9
Cytochrome *c* (33 μg/ml)	7
Lysozyme (33 μg/ml)	7
None	7

These assembly assays were performed as described in *Materials and Methods* except that histones were omitted and the indicated components were included in their place. In all cases the DNA was mixed with the extract prior to the addition of either histones or the indicated replacement.
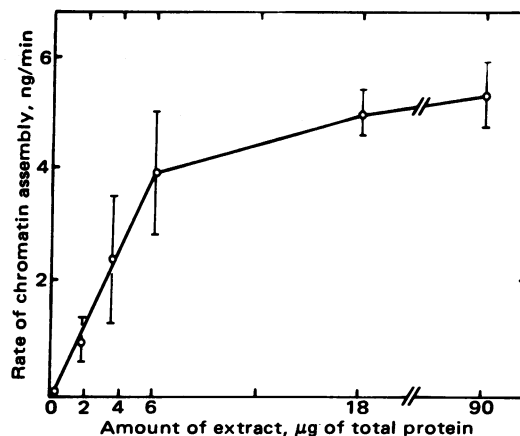

FIG. 6. Dependence of rate of assembly on amount of extract. Five micrograms of *E. coli* [^{3}H]DNA (5000 cpm/μg) was incubated with 5 μg of core histones and with varying amounts of the embryo extract containing 0–90 μg of total protein. Rates of formation of micrococcal nuclease-resistant DNA were determined from aliquots taken during the initial linear portion of the reaction (<10 min). The error bars represent the 95% confidence limits (mean ± 2 SD) on the initial rate.

7). Although the stimulation of the rate varied from extract to extract, the extent of the reaction was always increased 2-fold. Preincubation of the DNA for 10 min was found sufficient for optimal stimulation. Preincubation of the histones with the extract prior to the addition of DNA did not show any effects on either the rate or extent of chromatin assembly. The product of this two-stage reaction met all the criteria for proper nucleosome structure described above and differed from that of the original assembly assay only in amount. The stimulatory effect of preincubation of DNA with the extract suggests that the assembly occurs in two steps. First the extract interacts with the DNA, and then this modified or complexed DNA combines with histones in the actual assembly stage.
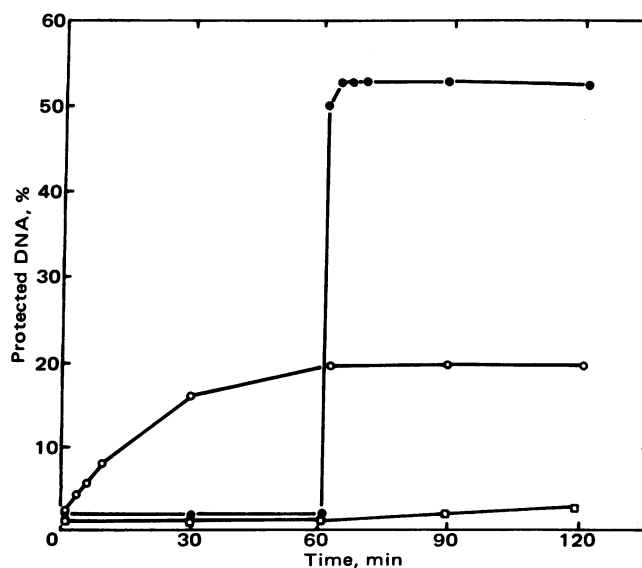

FIG. 7. Preincubation of DNA with extract stimulates rate and increases extent of chromatin assembly. Two-hour time course reactions were performed as described in the legend to Fig. 5 with the complete assembly reaction (O), with one in which the histones were omitted for the first 60 min (●), or with one in which both histones and the extract were omitted and histones alone were added after 60 min (□).

DISCUSSION

We have shown that an extract of *Drosophila* embryos, 1 hr after fertilization, is capable of converting exogenous DNA into a chromatinlike state as judged by several criteria. Covalently closed circular DNA is converted into a supercoiled form upon incubation with the extract. The protein–DNA complex formed contains micrococcal nuclease-sensitive sites at 200-base pair intervals. The embryo extract is also capable of assembling exogenous core histones (H2A, H2B, H3, and H4) and linear DNA into a chromatin-like structure *in vitro*. Micrococcal nuclease treatment of histone–DNA complexes formed with exogenous histones results in 11S particles that cosediment with nucleosome core particles isolated from native chromatin. These core particles contain 140-base pair DNA fragments which upon further digestion with micrococcal nuclease give rise to a set of discretely sized DNA fragments similar in length to the subnucleosomal fragments derived from digestion of native chromatin. The chromatinlike product assembled by this *Drosophila* embryo extract is similar to that produced in soluble extracts from *Xenopus* eggs (9).

Preincubation of DNA with the extract results in a 2-fold increase in the extent of chromatin assembly as well as a stimulation of the rate. Incubation of the histones with the extract shows no such stimulatory effect. These results indicate that components in the extract may be interacting first with the DNA and suggests a different assembly mechanism than that proposed by Laskey *et al.* (9, 10). They have demonstrated that extracts of *Xenopus* embryos contain an acidic protein which associates first with histones and that this complex then interacts with the DNA during chromatin assembly. Our results are similar to those of Germond *et al.* (23) who have demonstrated that chromatin assembly can be mediated by the nicking–closing enzyme, an enzyme isolated from chromatin itself, which interacts strongly with DNA. The extract of early embryos is known to contain large amounts of nicking–closing activities (unpublished observations). Our current results suggest that the factors responsible for promoting the orderly assembly of histones and DNA into nucleosomes first interact with DNA, perhaps by forming a protein–DNA complex which could then serve as a nucleation site for the addition of histones. Once formed, this hypothetical complex would allow the rapid assembly of exogenous histones into a chromatinlike structure.

All of the models suggested for the assembly of chromatin from histones and DNA *in vitro* are consistent with the inhibition or competition of the strong ionic interaction between histones and DNA. In order to allow proper folding of the DNA about the histone core it is probably necessary to inhibit nonspecific associations. Reconstitution of chromatin by dialysis from high salt minimizes such ionic interactions. The acidic histone-binding protein in *Xenopus* could compete for interaction with the DNA. Similarly, the binding of the nicking–closing enzyme or other factors to DNA might compete for histone binding. We have not been able to detect a thermostable or trypsin-resistant assembly factor that promotes assembly similar to that described in *Xenopus* (10).

Assay of chromatin assembly by the conversion of labeled linear DNA into a micrococcal nuclease-resistant form allowed a quantitative measure of both initial rates as well as limited extents of chromatin assembly. The DNA protection assay is also a very rapid quantitative method which does not depend on covalently closed circular DNA and does not require a relaxation step in order to detect chromatin assembly. The major disadvantage of the DNA protection assay is its inability to distinguish specific chromatinlike structures and nonspecific protection of DNA by histones. To minimize such nonspecific protection we have usually kept the amounts of histones below the amounts of DNA by weight. Higher amounts of histones will lead to both precipitation of the DNA under the conditions of the assay and higher backgrounds (5–10%). We have noticed that these high backgrounds in the presence of excess histones can be markedly decreased if the DNA is mixed with the extract prior to the addition of the histones. Only this order of addition allows the assay of chromatin formation in the presence of histone excess without precipitation of the DNA. This observation again suggests that the extract can interact first with the DNA to inhibit nonspecific histone–DNA associations.

The initial rate of chromatin assembly observed *in vitro* (10 ng of DNA per min per embryo) is consistent with the maximal rate of chromatin assembly required of preblastoderm embryos (1–2 ng of DNA per min per embryo). The specific activity of these embryo extracts for chromatin assembly (0.67 ng of DNA per min per μg of extract protein) is as great as the activity obtained from chromatin extracts (1 ng of DNA per min per μg of extract protein) and much more active than extracts of *Xenopus* eggs (0.005 ng of DNA per min per μg of extract protein) (9). These results are consistent with the rapid rates of DNA replication observed in *Drosophila* embryos (11).

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