Methods in Cell Biology

Prepared under the Auspices of the American Society for Cell Biology

VOLUME 44

Drosophila melanogaster:
Practical Uses in Cell and Molecular Biology

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ACADEMIC PRESS
San Diego New York Boston London Sydney Tokyo Toronto

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CHAPTER 12

Chromatin Assembly Extracts from *Drosophila* Embryos

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METHODS IN CELL BIOLOGY, VOL. 44

I. General Introduction

A deeper understanding of the major processes that constitute nucleic acid metabolism, replication, transcription, recombination and DNA repair, requires the reconstitution of these phenomena *in vitro* in the context of chromatin, their natural substrate. The first level in the hierarchy of chromatin folding, the association of DNA with the core histones to form nucleosomes and the binding of the linker histone H1, is currently amenable to *in vitro* reconstitution. One salient feature of natural chromatin, the retention of a defined distance between nucleosome core particles in an extended array of nucleosomes (nucleosome spacing), can be reconstituted only under physiological conditions using crude, chromatin assembly extracts derived from tissue culture cells (Banerjee and Cantor, 1990), *Xenopus* eggs and oocytes (Almouzni and Méchali, 1988; Shimamura *et al.*, 1988), and *Drosophila* embryos (Becker and Wu, 1992). Unlike extracts prepared from *Xenopus* eggs or oocytes, which are sometimes subject to seasonal variation (Rodríguez-Campos *et al.*, 1989), extracts prepared from *Drosophila* embryos harvested from population cages reproducibly yield extracts of high activity. As *Drosophila* can be raised cheaply in mass culture requiring minimal attention, large quantities of staged embryos can be harvested routinely in amounts sufficient for biochemical manipulations (see Chapters 5, 7, and 10). In addition, extracts of fly embryos are a rich source of various biochemical activities, including factors necessary for *in vitro* transcription (see Chapter 13).

Nelson, Hsieh, and Brutlag (1979) initially exploited *Drosophila* embryos as a source of extracts for chromatin assembly. While extracts prepared according to their procedure were capable of assembling nucleosomes on plasmid DNA, the assembled nucleosomes, in our experience, lacked defined spacing in extended nucleosome arrays and these extracts lost activity upon storage. In exploring alternative procedures for the preparation of chromatin assembly extracts, we found that ionic conditions developed for extract preparation from the *Xenopus* oocyte system (Shimamura *et al.*, 1988, Rodriguez-Campos *et al.*, 1989) yielded a sturdy reconstitution extract with consistently high activity for the assembly of long arrays of spaced nucleosomes (Becker and Wu, 1992).

The *Drosophila* chromatin assembly extracts are essentially cytoplasmic supernatants generated by centrifugation of embryo homogenates in a low ionic strength buffer at 150,000 g (S-150). The assembly extracts are prepared from preblastoderm embryos [collected from a window of 0–90 min, or 0–120 min after egg laying (AEL)]. These early embryos, which are largely inactive for transcription, undergo DNA replication and chromatin assembly at maximal rates, relying on stores of maternal precursors. Chromatin reconstitution *in vitro* with the S-150 extracts utilizes this endogenous pool of maternal histones and should therefore resemble preblastoderm chromatin. The chromatin of preblastoderm embryos differs from that of later developmental stages in that the linker histone H1 is absent or substituted with an as yet undefined, alter-

nate linker histone. Kamakaka *et al.* (1993) have employed similar ionic conditions to prepare chromatin assembly extracts from slightly older embryos (0–6 hr AEL). At this stage, the maternal pool of stored histones is exhausted, and these extracts require supplementation with exogenous, purified core histones. Both preblastoderm and postblastoderm extracts contain little histone H1. However, exogenous H1, purified from late embryo chromatin can be introduced in the assembly reaction, where it becomes incorporated into chromatin and increases the nucleosome repeat length from ~180 to ~200 bp.

Cytoplasmic extracts of the kind described here contain high levels of enzymes and cofactors required for DNA synthesis and are thus capable of synthesizing the DNA strand complementary to a single-stranded (ss) circular DNA template; this reaction is also accompanied by chromatin assembly (Becker and Wu, 1992; Kamakaka *et al.*, 1993). The extracts can therefore be used to perform coupled replication/assembly reactions similar to those shown with extracts of Xenopus eggs (Almouzni *et al.*, 1990) and tissue culture cells (Krude and Knippers, 1993). The coupled replication/assembly reactions may more closely approximate the physiological deposition of histones on DNA during replication *in vivo*.

In this chapter, we describe protocols for the preparation of chromatin assembly extracts from *Drosophila* embryos and procedures for the assembly of regularly spaced nucleosomes on plasmid DNA and for the analysis of the resulting chromatin.

II. Extract Preparation

The preparation of active chromatin assembly extracts according to Becker and Wu (1992) is schematically described in Fig. 1. Briefly, dechorionated embryos are homogenized with minimal dilution in a low salt buffer. The crude homogenate is supplemented with additional $MgCl_2$ before the embryonic nuclei are pelleted by low-speed centrifugation. The cytoplasmic extract is then cleared by centrifugation at high speed (150,000*g*), which effectively removes (floating) lipids and pellets the yolk granules, organelles, and other cellular debris. This clarified supernatant (S-150) is the chromatin assembly extract. We have prepared over one hundred S-150 extracts in our laboratories; the extracts have consistently high activity and are stable upon storage for many months and after several cycles of freeze and thaw.

A. Preparation of Extracts from Preblastoderm Embryos

Embryos (0–90 min or 0–120 min (preblastoderm) are harvested from three population cages each containing 50,000 flies maintained on a 12-hr day/night cycle. To purge older embryos retained by females during the overnight period, embryos deposited in the first hour of the daily collection are discarded.

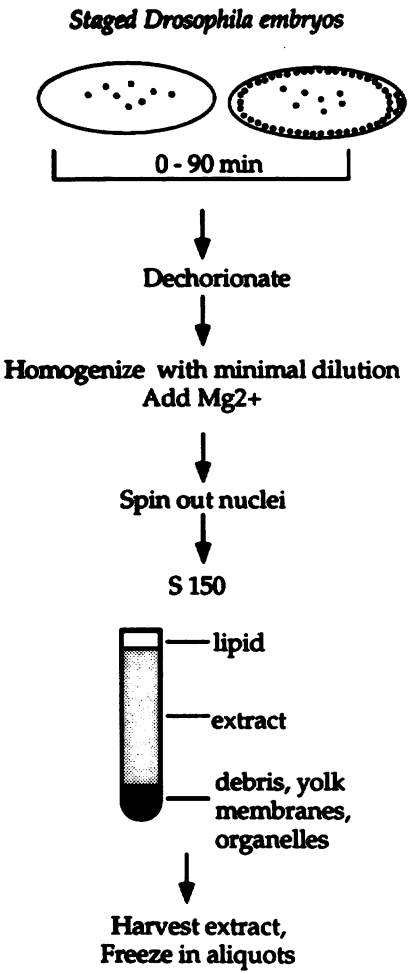

Fig. 1 Schematic representation of the steps required for preparation of chromatin assembly extracts from *Drosophila* embryos.

Throughout the day, embryos are harvested in successive 90- or 120-min intervals and stored in embryo wash buffer (0.7% NaCl, 0.05% Triton X-100) on ice, where further development is arrested. Pooled embryos are allowed to settle once in 1.0-liter embryo wash buffer at room temperature, and the volume of the suspension is adjusted to 200 ml. Dechorionation occurs by either adding 200 ml of Clorox bleach and vigorous stirring for 90 sec (USA) or adding 60 ml 13% hypochloric acid and stirring for 2.5–3 min (Europe). Embryos are collected on a fine sieve and rinsed vigorously with a sharp stream of tap water. They are then allowed to settle once or twice in at least 500 ml of embryo wash buffer, and the supernatant containing broken chorions is removed by

aspiration. The substantial loss of material due to floating embryos in this and the following washes is usually a sign of insufficient dechorionation or inadequate rinsing.

The embryos are resuspended and allowed to settle in 500 ml 0.7% NaCl, followed by resuspension and settling in 350 ml cold EX buffer (10 m*M* Hepes, pH 7.6/10 m*M* KCl/1.5 m*M* $MgCl_2$/0.5 m*M* EGTA/10% glycerol/10 m*M* β-glycerophosphate) to which 1 m*M* dithiothreitol and 0.2 m*M* phenylmethylsulfonyl fluoride has been added freshly. Finally, they are transferred to a 60-ml glass homogenizer containing EX buffer and allowed to settle for 15 min on ice. We routinely obtain ~20 ml of packed embryos from the pool of four successive embryo collections. The supernatant is aspirated, leaving behind about 2 ml of buffer on the surface of the packed embryos. All further manipulations are performed at 4°C.

Embryos are homogenized by 6–10 complete strokes at 1500 rpm using a teflon pestle connected to a motor-driven drill press (or 1000 rpm in a B. Braun homogenizer). The volume of the homogenate is determined and $MgCl_2$ is quickly mixed in from a 1 *M* stock solution to increase the $MgCl_2$ concentration by 5 m*M* to a final concentration of 6.5 m*M*. This concentration of $MgCl_2$ has not been optimized, and a final concentration of 5 m*M* $MgCl_2$ has also been found adequate (T.T.). Nuclei are then pelleted by centrifugation for 5 min at 5000 rpm in a chilled rotor HB4 (Sorvall) or JA14 (Beckmann). The supernatant is clarified by centrifugation for 2 hr at 150,000*g* (40,000 rpm in a SW 50.1 rotor; Beckman or equivalent).

The homogenate splits into three fractions after centrifugation, comprising a solid pellet, a mostly clear supernatant, and a floating layer of lipid (see Fig. 1). The clear extract is collected with a syringe by puncturing the tube with the syringe needle just above the tight pellet, thus avoiding the floating lipid layer. Occasionally, a white flocculant material is present in the otherwise clear extract. Much of this can be removed by centrifugation for 5 min in an Eppendorf microcentrifuge. The presence of some turbid material in an extract appears to be correlated with insufficient dechorionation. It usually does not affect the chromatin assembly reaction but may result in an increased background of copurifying proteins when reconstituted chromatin is partially purified on sucrose gradients. The S-150 extracts are flash-frozen in suitable aliquots and stored at −80°C. They can be thawed and refrozen two or three times and stored at −80°C for a year without significant loss of activity. The protein concentration of these extracts is usually around 20 mg/ml.

B. Preparation of Extracts from Postblastoderm Embryos

Kamakaka *et al.* (1993) have reported a procedure similar to the protocol described above to prepare S-190 extracts for chromatin assembly from slightly older embryos (0–6 hr AEL; mostly postblastoderm embryos). We have prepared S-190 extracts from 0- to 6-hr embryos using the above protocol and

concur with their results. Kamakaka *et al.* (1993) have noted that additional precipitation of material is observed upon freezing and thawing of the S-190 extract; this material is pelleted by recentrifugation at 45,000 rpm for 2 hr. Although this additional step appears not to be necessary for the proper assembly of nucleosomes per se, it may have important consequences depending on the desired functional assay for the reconstituted chromatin and may therefore be incorporated in the overall protocol by the user.

While the properties of nucleosome structure reconstituted with extracts of preblastoderm and postblastoderm embryos should be similar at a gross level, it is likely that the chromatin assembled using these differently staged embryo extracts may possess differences in histone modification peculiar to each embryonic stage. These and other potential differences should be taken into consideration when evaluating the structural and functional properties of the reconstituted chromatins.

III. Chromatin Assembly Reaction

Chromatin assembly in the S-150 (or S-190) embryo extract is usually performed at 26–27°C, near the optimal temperature for *Drosophila* development. The reaction also occurs efficiently at slightly elevated temperatures (30°C). The assembly of regularly spaced nucleosomes requires magnesium, ATP, an energy regenerating system, and a defined concentration of monovalent cations. The conductivity of a standard assembly reaction is equivalent to 65 m*M* KCl. The optimal amount of extract needed for the assembly of spaced chromatin is determined empirically by a micrococcal nuclease (MNase) digestion assay and is roughly similar when different extract preparations are compared.

A. Chromatin Assembly Using Preblastoderm Embryo Extracts

We present conditions for the assembly of 900 ng plasmid DNA; the reaction can be scaled up or down 10-fold.

Prepare 10× MCNAP buffer for energy regeneration by adding:

46 μl H_2O

30 μl 1 *M* creatine phosphate (in water)

10 μl 300 m*M* ATP, pH 7.0

10 μl 100 ng/μl creatine phosphokinase (Sigma)

3 μl 1 *M* $MgCl_2$

1 μl 1 *M* DTT

(Creatine phosphate, creatine phosphokinase, and ATP should be stored in aliquots at −80°C and thawed only once before use).

Combine in a 1.5 ml reaction tube:

12 μl 10× MCNAP buffer
108-x-y μl EX buffer/50 m*M* KCl (EX 50)
y μl extract (~75 μl; titrate for each extract preparation)
x μl DNA (900 ng)
Incubate at 26°C for up to 6 hr.

B. Incorporation of Exogenous Histone H1

Extracts from preblastoderm embryos contain very little of the major linker histone H1 and no H1 is detected in reconstituted chromatin. When purified H1 is added to the assembly reaction, it is incorporated into chromatin. The binding to linker sequences results in an increased repeat length in a micrococcal nuclease digestion analysis (see following, Fig. 3B). Histone H1 is easily purified from late embryo chromatin using the protocol of Croston *et al.* (1991). We dilute purified histone H1 with EX buffer containing 0.01% NP-40 to prevent aggregation and mix it with the assembly extract prior to addition of the DNA. Given the difficulties in determining the precise concentrations of histone H1 with standard dye-binding assays, we empirically titrate the amounts of H1 required to increase the nucleosome repeat length from (~180 to ~200 bp (Fig. 3B). If excess H1 is added, it competes with the core histones in binding to DNA, leading to improper nucleosome spacing.

C. Chromatin Assembly Using Postblastoderm Embryo Extracts and Exogenous Histones

Exogenous histones can be used for chromatin reconstitution in conjunction with extracts from postblastoderm embryos that have depleted the maternal pools of histones (Kamakaka *et al.*, 1993). Core histones are purified according to the method of Simon and Felsenfeld (1979) but commercially available calf thymus histones (Boehringer Mannheim, Catalog No. 223 656) can also be used. The appropriate amount of histones is determined empirically, using as a guide a stoichiometry of histones to DNA of ~0.8 : 1 (w/w) (Albright *et al.*, 1979). The following protocol assembles 900 ng of DNA in chromatin.

Combine and incubate for 20–30 min at 26–27°C (to ensure that histones associate with carrier molecules in the crude extract):

55 μl extract from 0- to 6-hr embryos
1 μl of core histones (amount determined by titration)

Add to this mixture:

7 μl of 10× MCNAP buffer
900 ng of plasmid DNA

Adjust the volume to 70 μl with EX 50 buffer and incubate at 26°C for 1 to 6 hr. Properly spaced chromatin should be assembled by 3 hr of incubation, as visualized by the ladder of DNA fragments produced by partial MNase digestion.

D. Coupled Replication/Chromatin Assembly

If the double-stranded (ds) DNA in the assembly reaction is replaced by ss DNA, the complementary strand will be synthesized by the DNA replication enzymes that are abundant in the S-150 or S-190 extracts. To exploit this reaction for coupled replication/assembly, the DNA template is cloned into a phagemid such as pBluescript (Stratagene, La Jolla, CA), and ssDNA is obtained according to standard procedures (Sambrook *et al.*, 1989).

The protocol for the coupled replication/assembly reaction is similar to the standard reaction for dsDNA except that the 900 ng of plasmid is replaced by 450 ng of ss DNA. If random labeling of the resulting dsDNA plasmid is desired, 1 μl of [α^{32}P]dCTP (NEN, 2000- 3000 Ci/mmole) is included. Priming of DNA synthesis is random, presumably from RNA primers synthesized in the extract, but for the purpose of site-specific labeling, terminally labeled oligonucleotides can be incorporated into the resulting plasmid if annealed to the ss DNA prior to addition to the assembly reaction (Becker and Wu, 1992; Kamakaka *et al.*, 1993).

IV. Analysis of Reconstituted Chromatin

We describe two standard procedures for the initial characterization of reconstituted chromatin that are also useful to monitor the efficiency of an assembly reaction. The supercoiling assay is based on topological changes that accompany the wrapping of DNA around a particle. The winding of the DNA around a nucleosome core introduces one positive superhelical turn in the plasmid DNA, which is relaxed by topoisomerase I activity present in the embryo extracts. When nucleosomes are removed by proteinase K digestion and DNA purification, one negative superhelical turn corresponding to each assembled nucleosome appears in the closed circular DNA (Germond *et al.*, 1975). The superhelical density of a plasmid, i.e., the absolute number of superhelical turns, can be directly counted by visualization of the plasmid topoisomers on two-dimensional agarose gels (Peck and Wang, 1983) or by resolving duplicate samples on multiple agarose gels containing different chloroquine concentrations (Keller, 1975). As a rapid, but crude indicator of nucleosome reconstitution, the introduction of supercoils into a plasmid can simply be visualized by agarose gel electrophoresis (Fig. 2A).

DNA supercoiling measures the wrapping of DNA around a particle but does not necessarily imply the reconstitution of a full octamer of core histones.

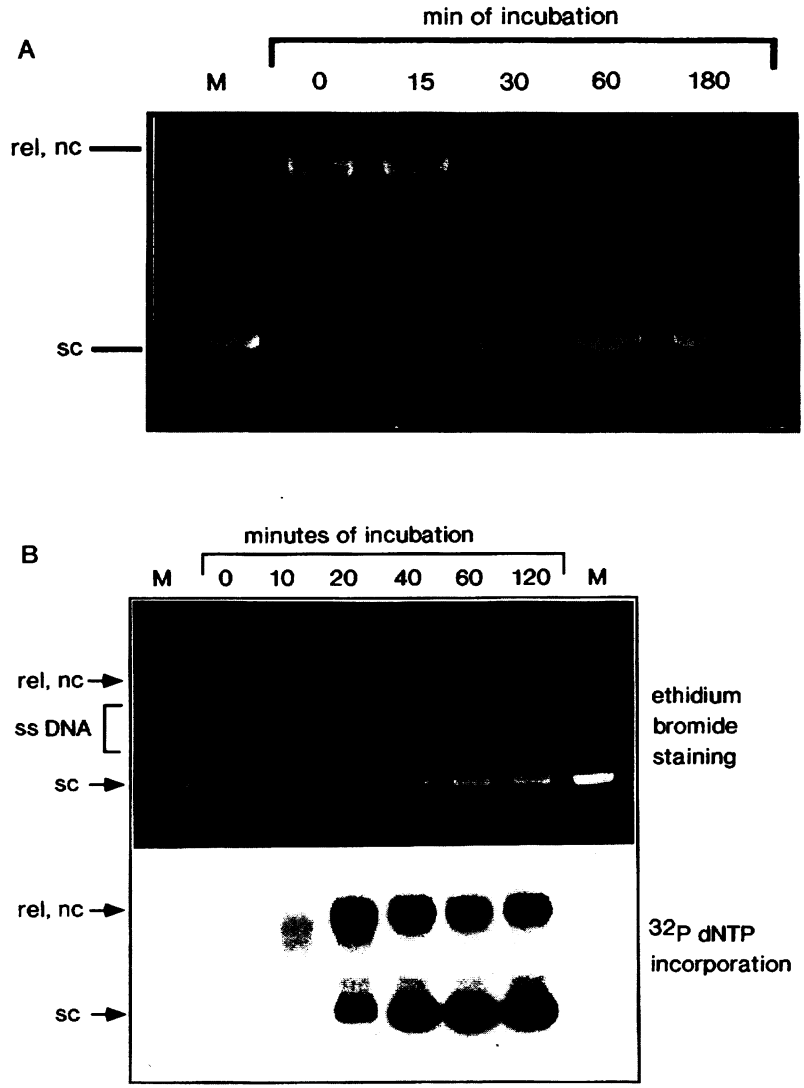

Fig. 2 (A) DNA supercoiling assay for assembled chromatin. 1.5 μg of plasmid DNA was relaxed with topoisomerase I and then incubated with 120 μl preblastoderm embryo extract under assembly conditions. At the times indicated, 40-μl aliquots were removed and the DNA was purified for analysis on a 1.2% agarose gel. Sc, supercoiled DNA (form I); rel, relaxed, closed circles (form II); nc, nicked circles. (B) Supercoiling assay showing chromatin assembly coupled with DNA synthesis in embryo extracts. Duplicate reactions containing 150 ng of ss phagemid DNA (6.2 kb) were incubated under assembly conditions in the presence of 0.5 μCi of [^{32}P]dCTP with 15 μl preblastoderm extract in a volume of 30 μl. Reactions were terminated at the indicated times and analyzed for supercoiling on an 0.8% agarose gel. DNA was visualized by staining with ethidium bromide (upper panel). The gel was then blotted and dried onto DE81 paper (Whatman). The lower panel shows an autoradiography of the dried gel.

Indeed, the winding of DNA around a complete histone octamer or a tetramer of histones H3 and H4 cannot be distinguished by this method. The MNase digestion assay, although more time consuming, is much more informative because it provides information on the nature of the nucleosome core particle as well as on the average distance between particles. This assay relies on the ability of MNase to preferentially cleave the linker DNA between nucleosome core particles. After the initial endonucleolytic attack of linker DNA, the trimming activity associated with enzyme progressively removes the linker DNA. Extensive digestion of chromatin with MNase will bring the size of the mononucleosome from 160–220 bp to the 146-bp DNA fragment protected by the nucleosome core particle (Bavykin *et al.*, 1990) whereas a partial digest results in a ladder of fragments representing oligonucleosomal DNAs (Fig. 3).

When the extent of chromatin reconstitution in the course of an assembly reaction is analyzed by measuring DNA supercoils, the rate at which supercoils are introduced is rapid: about 80% of the maximal number of supercoils are introduced within the first 30–60 min of incubation (Fig. 2A). By contrast, a regular pattern of digestion with MNase is obtained only after the reaction is allowed to proceed for an extended period of time. An appreciable improvement in quality of the MNase digestion ladder is observed when a 6-hr incubation is compared with a 3-hr incubation, even though few additional supercoils are introduced during this time interval. It is possible that the rapid introduction of DNA supercoils reflects the early assembly of subnucleosomal particles (H3–H4 tetramers) and that the complete assembly of the histone octamer requires an extended period of incubation *in vitro*.

A. Supercoiling Assay

We allocate 150–300 ng of plasmid DNA for each time point to be analyzed. A total of 1.5 μg plasmid DNA are incubated under assembly conditions (see Section IIA) with 120 μl of chromatin assembly extract in a total volume of 200 μl. After 15, 30, 60, and 180 min of incubation at 26°C, 40-μl aliquots of the reaction are added to 10 μl Stop Mix (2.5% *N*-lauroylsarcosine (Sigma), 100 m*M* EDTA).

The purification of the DNA essentially follows the procedure of Shimamura *et al.* (1988). Each sample is incubated with 1 μl of 10 mg/ml RNase A (DNase-free; Sambrook *et al.*, 1989) for 15 min at 37°C. Then, 6.5 μl of each of 2% SDS and 10 mg/ml proteinase K are added, and incubation at 37°C is continued for 30 min. The reaction is adjusted to 3*M* ammonium acetate by addition from a 7.5 *M* stock solution, 10 μg glycogen (Boehringer Mannheim) is added, and the sample is mixed. After addition of 2 vol of ethanol, DNA is precipitated for 15 min on ice. The addition of glycogen helps visualization of the pellet but is not generally required for nucleic acid precipitation. After centrifugation for 10 min in a microcentrifuge, the pellet is washed with 1 ml of 80% ethanol. The ethanol is removed completely and the DNA is dried for 3 min in a Speed

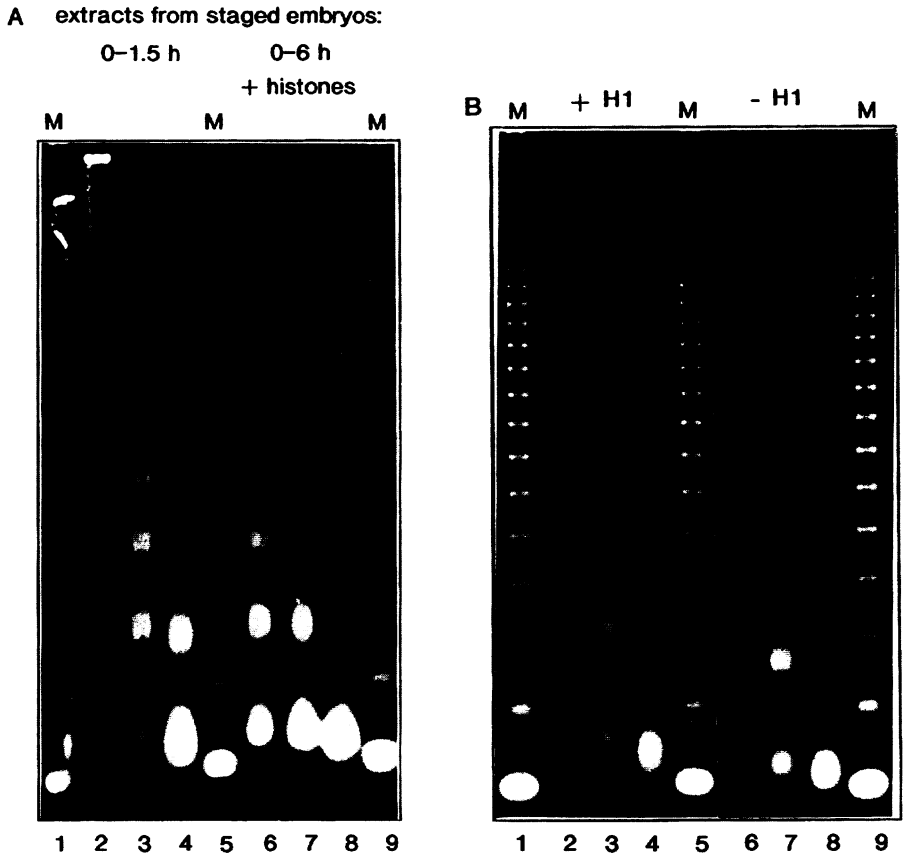

Fig. 3 (A) Micrococcal nuclease digestion assay of assembled chromatin. Nine hundred nanograms of plasmid DNA, assembled into chromatin in extracts from 0- to 2-hr embryos (lanes 2–4) or 0- to 6-hr embryos with supplemented histones (lanes 6–8) were analyzed by digestion with micrococcal nuclease as described. Purified DNA fragments were resolved in a 1.3% agarose gel stained with ethidium bromide. M, 123-bp ladder (BRL). (B) Incorporation of exogenous histone H1 in assembled chromatin. Chromatin was reconstituted in an extract from 0- to 90-min embryos in the presence (lanes 2–4) or absence (lanes 6–8) of H1. The MNase assay reveals an increase in repeat length upon incorporation of H1.

Vac concentrator. The DNA pellet is dissolved in 8 μl TE (10 m*M* Tris/Cl, pH 8.0; 1 m*M* EDTA), 2 μl of (5×) blue loading buffer (30% glycerol, 0.25% each of bromophenol blue and xylene cyanol) is added, and the sample is analyzed on an agarose gel in 1× Tris-glycine buffer (5× buffer: 144 g glycine, 30 g Tris/liter). The entire electrophoresis apparatus should be kept clean of ethidium bromide because intercalation of the dye during electrophoresis will cause additional DNA supercoiling (Keller, 1975). The agarose concentration of the gel is determined by the plasmid size (we use 1.2% agarose gels for 3- to 4-kb

plasmids, and 0.8% agarose gels for 6- to 7-kb plasmids). The best resolution of topoisomers is achieved during overnight runs at 1 V/cm; however, satisfactory results are obtained at up to 5 V/cm. After electrophoresis, the gel is stained for 15–30 min in 1 gel volume of water containing 2 μg/ml ethidium bromide and destained for 15 min in deionized water.

Figure 2A shows a typical supercoiling assay. For better illustration of supercoiling, 1.5 μg of plasmid DNA was previously relaxed with topisomerase I (purified according to Javaherian *et al.*, 1982) in a total volume of 60 μl of EX-50/0.05% NP40 (Fig. 2A, 0 min). This prerelaxation is generally not required in practice, since topoisomerase I activity in embryo extracts almost immediately relaxes supercoiled plasmid DNAs upon incubation in the assembly reaction. After 30 min of incubation in the assembly extract, the purified plasmid DNAs are observed to be highly supercoiled again. The resolution of topoisomers of higher superhelical densities requires electrophoresis in the presence of chloroquine (Peck and Wang, 1983).

Figure 2B shows a supercoiling assay of a reaction starting from ss DNA in a coupled DNA synthesis/assembly reaction (Section IIID). The ss DNA (0 min incubation) is converted into supercoiled ds DNA in a reaction that is essentially complete by 30–40 min (top panel). If the histones are removed from such a reaction prior to addition of the ss DNA, relaxed plasmids are obtained, indicating that the DNA synthesis reaction can be uncoupled from the nucleosome assembly reaction (P.B.B., unpublished observations). The incorporation of [^{32}P]dCTP during the synthesis of the second strand can be followed by exposure of the dried gel to X-ray film (Fig. 2B, lower panel).

B. Micrococcal Nuclease Digestion

Nine hundred nanograms of plasmid DNA is assembled into chromatin as described (Section IIA). After incubation for 5–6 hr at 26°C, 180 μl of a premix containing 168 μl EX buffer, 9 μl 0.1 *M* $CaCl_2$, 3 μl MNase (50 u/μl) is added and the samples are again incubated at 26°C. (The concentration of $CaCl_2$ can also be decreased by half; T.T.) After 0.5, 1, and 5 min of incubation, a 100-μl aliquot is added to a fresh tube containing 25 μl Stop Mix (2.5% *N*-laurylsarcosine (Sigma), 100 m*M* EDTA). One microliter of 10 mg/ml RNase A is added and the reaction is incubated for 30 min at 37°C. The reaction is adjusted to 0.2% SDS and 300 μg/ml proteinase K and incubated overnight at 37°C. DNA is precipitated, pelleted, and washed as described above for the supercoiling assay (IVA). All traces of ethanol are removed and the pellets are air dried for 15–20 min on the bench. The pellets are dissolved in 4 μl of TE/50 m*M* NaCl. One microliter of loading buffer (50% glycerol/5 m*M* EDTA/0.3% orange G (Sigma) is added to each sample, and the samples are electrophoresed on a 1.3% agarose gel in Tris/glycine buffer. Superior resolution of long oligonucleosomal fragments can be achieved with narrow gel slots (Shimamura *et al.*, 1988). Samples are electrophoresed at 3 V/cm until the orange dye reaches the bottom

of the gel, and the gel is stained with ethidium bromide as above (Section IVA). Alternatively, DNA is electrophoresed on a 1.3% agarose gel in 0.5× TBE at 7 V/cm until the Orange G dye has migrated 10 cm.

Figure 3A shows the result of MNase digestions of chromatin reconstituted with a preblastoderm embryo extract (lanes 2–4) or with an extract of postblastoderm embryos and supplemental core histones (lanes 6–8). The nucleosome repeat lengths are determined by comparison of the largest visible oligonucleosome-sized fragment with the marker DNA fragments (123-bp ladder, BRL; see Rodriguez-Campos *et al.* (1989) for a discussion of how the repeat lengths are determined). The introduction of an appropriately titrated amount of histone H1 to the assembly extract prior to the addition of the DNA results in an increased repeat length in the MNase digestion assay (Fig. 3B; compare lanes 2–4 to lanes 6–8).

V. Nucleosome Organization at Specific Sites

The presence of nucleosome organization at specific locations on the recombination DNA clone is analyzed by Southern blotting of the DNA fragments produced by digestion with MNase followed by hybridization with unique oligonucleotide probes. Since MNase initially cleaves within the stretch of linker DNA between nucleosome core particles, followed by progressive trimming to the core from both ends of the nucleosome, the presence of a nucleosome core particle can be gauged by the accumulation of the canonical, 146-bp resistant fragment towards the limit of MNase digestion.

Figure 4 shows the assembly of an intact nucleosome at sequences corresponding to −115 to −132 of the *Drosophila* hsp70 promoter, to the 3′ end of the hsp70 gene, and to the ampicillin resistance gene of the plasmid vector. In addition to the 146-bp fragment derived from the nucleosome core particle observed after extensive MNase digestion, a ladder of discrete fragments corresponding to nucleosome oligomers can be observed at intermediate stages of MNase digestion. This characteristic pattern of cleavage indicates that those DNA sequences are organized in a regularly spaced (but not necessarily positioned) array of nucleosomes. The determination of nucleosome positioning and nuclease hypersensitive sites in chromatin can be revealed by the technique of indirect end-labeling (for technical protocols, see Wu, 1989).

A. Southern Blotting

The DNA products of MNase digestion are electrophoresed along with radiolabeled DNA markers on agarose gels as described above (Section IVB). The gel is treated for 45 min in 0.5 *M* NaOH, 1.5 *M* NaCl at room temperature with constant agitation to denature DNA. After briefly rinsing with water twice, neutralize the gel for 45 min in 1 *M* Tris–HCl, pH 7.5, 1.5 *M* NaCl at room

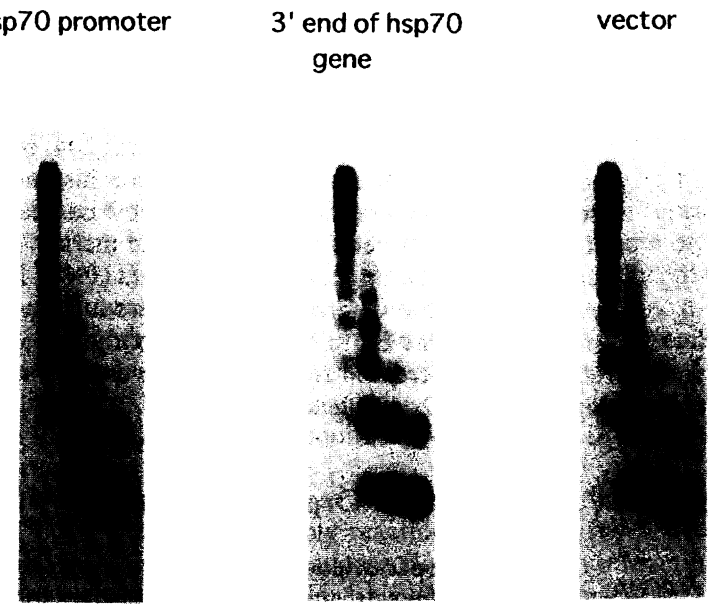

Fig. 4 Nucleosome organization at specific sites on a 6-kb *hsp70* plasmid as revealed by sequential oligonucleotide hybridization. The same DNA blot of a MNase digestion series was sequentially hybridized with oligonucleotides corresponding to −115 to −132 of *hsp70* promoter, +1803 to +1832 of the *hsp70* gene, and 2499 to 2528 of the pBluescript SK- vector.

temperature with constant agitation. DNA is transferred onto the hybridization membrane by capillary blotting overnight (Sambrook *et al.*, 1989). For the purpose of sequential hybridization with oligonucleotide probes (see following), we use nylon membrane without a surface charge (Gene Screen, DuPont/NEN) because of the ease of handling and low-background signals. DNA fragments are fixed onto the membrane by cross-linking with UV light, according to the manufacturer's instructions.

B. Oligonucleotide Hybridization

We routinely use oligonucleotide probes 17 to 40 bases in length. Usually, purification of oligonucleotides by sequencing gel electrophoresis or HPLC is not required. Oligonucleotides are 5′ end-labeled as follows:

5 pmole of oligonucleotide
1.5 μl of 10× T_4 polynucleotide kinase buffer (Sambrook *et al.*, 1989)
1 μl of [γ-^{32}P]ATP (7000 Ci/mmole) 166.7 μCi/μl, ICN Catalog 35020)
1 μl (10 u) of T_4 polynucleotide kinase
Make up to 15 μl with water

Incubate the reaction mixture at 37°C for 45 to 60 min. Terminate the reaction by adding SDS to 1% and purify the oligonucleotide through a spin column (Bio-spin 6, Bio-Rad) according to the manufacturer's protocol.

Wet the DNA blot with water and prehybridize for >30 min in 6× SSC (1× SSC: 0.15 *M* NaCl, 0.015 *M* Na citrate), 2% SDS, 100 μg/μl denatured salmon sperm DNA at the hybridization temperature. After introducing the labeled oligonucleotide directly into the prehybridization mixture, allow the probe to hybridize for 2–10 hr at 40–55°C, depending on the T_m of the probe (we usually set the temperature at T_m − 10°C). Wash the membrane at hybridization temperature two to three times in 6× SSC, 0.5% SDS for 15–30 min each. The hybridization solution can be stored at 4°C and reused several times within a week. After wrapping with SaranWrap, expose the membrane to film for several hours to overnight. Be careful not to let the membrane dry out during exposure.

To strip off the probe for rehybridization, incubate the membrane in 0.5 *M* KOH at 40–50°C for 1 hr. If the background signal from the previous hybridization is high, we wash the membrane in the same solution overnight at 65°C. After rinsing with water, the membrane can be stored dry or it can be hybridized with another probe. We have successfully rehybridized the same membrane more than eight times.

VI. Conclusions and Perspectives

The *in vitro* chromatin assembly system from *Drosophila* embryos enables the reconstitution of cloned genes in chromatin with regularly spaced nucleosomes. As the reconstituted chromatin is transcriptionally repressed (Becker and Wu, 1992; Kamakaka *et al.*, 1993), this template approaches the *in vivo* structure of inert chromatin that is the substrate for interaction with transcription factors, RNA polymerase, and other sequence-specific-binding proteins. Thus, the ability to reconstitute transcriptionally repressed chromatin provides a starting point for investigations on the mechanism of action of these proteins in a near-physiological context. The assembly system should also be useful for the analysis of the pathway of histone deposition, for an analysis of the higher orders of chromatin folding, and more generally for mechanisms of DNA replication and recombination in a chromatin context. The evolutionary conservation of core histone structures, and the feasibility of incorporating the species-specific linker histone H1 exogenously suggest that the *Drosophila* system may additionally serve to mimic the chromatin structure of mammalian genes.

While the present *in vitro* system is efficient and reliable, there is the disadvantage of working with a crude, unfractionated extract. Hence, a considerable challenge for the future will be the purification and characterization of the individual components required for the complex process of nucleosome assembly and spacing. Notwithstanding the lack of a purified system, the crude extract has proved useful in addressing the question of nucleosome positioning on a

mammalian α-fetoprotein gene (McPherson *et al.*, 1993) and in the mechanism of nucleosome disruption by a constitutively active GAGA transcription factor (Tsukiyama *et al.*, 1994). A further elaboration of the crude system toward the assembly of chromatin on magnetic beads promises to greatly extend its utility for solid phase analyses (Sandaltzopoulos *et al.*, 1994). The *in vitro* assembly system may also aid biochemical studies of *Drosophila* mutants with phenotypes suggesting an involvement of chromatin structure, i.e., the suppressors and enhancers of position effect variegation (Shaffer *et al.*, 1993) and the *polycomb* and *trithorax* group genes (Paro, 1990; Tamkun *et al.*, 1992).

Acknowledgments

We acknowledge postdoctoral fellowship support from Deutsche Forschungsgemeinschaft (P.B.B.) and the NIH Fogarty Center (P.B.B. and T.T). This work was supported by the Intramural Research Program of the National Cancer Institute, National Institutes of Health.

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