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Ribonucleic Acid and Other Polyanions Facilitate Chromatin Assembly in Vitro†

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ABSTRACT: Crude extracts of *Drosophila* embryos are a rich source of both DNA topoisomerase I and chromatin assembly activity [Nelson, T., Hsieh, T., & Brutlag, D. L. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 5510–5514; Hsieh, T., & Brutlag, D. L. (1980) *Cell (Cambridge, Mass.) 21*, 115–125]. Purified topoisomerase I from *Drosophila* embryos, however, is not sufficient for chromatin assembly. Rather, the ability of *Drosophila* embryo extracts to mediate chromatin assembly in vitro requires an anionic fraction which we demonstrate to be RNA. Exogenous natural and homopolymer RNAs, if of sufficient length, can also mediate chromatin assembly in vitro. The RNA acts stoichiometrically in assembly, being required in amounts at least equal in weight to the amount of histones present. Natural and homopolymer DNAs, whether single or double stranded, are inactive under the same conditions. The arginine-rich histones H3 and H4 or histone H4 alone is sufficient to produce nucleoprotein complexes with physiological numbers of supertwists in the DNA. Complexes containing these subsets of the core histones also resemble assembled complexes containing all four core histones with respect to some patterns of nuclease sensitivity, although complexes containing all four core histones more closely resemble native chromatin in nuclease digestions.

While the structure of the basic repeating subunit of chromatin, the nucleosome, is understood in great detail (Kornberg, 1977; Felsenfeld, 1978; McGhee & Felsenfeld, 1980), how eukaryotic DNA is folded into this structure is largely unknown. Many features of nucleosomal structure can be generated from mixtures of DNA and histones in vitro by slow dialysis to physiological ionic conditions from 2 M NaCl, in which the correct histone octamers are stabilized and DNA–histone ionic interactions are neutralized (Felsenfeld, 1978). The strong ionic interactions between histones and DNA lead to the formation of nonspecific precipitates in simple mixtures at physiological ionic strengths (0.1–0.2 M NaCl).

Recently, several experimental systems have been described which permit chromatin assembly in vitro at physiological ionic strength. Laskey et al. (1977) have shown that extracts from unfertilized *Xenopus* eggs can assemble histones and SV40[1] DNA into minichromosomes. They have purified an acidic thermostable protein from these extracts that binds histones and transfers them to DNA (Laskey et al., 1978). The protein resembles the HMG class of chromosomal proteins (Laskey et al., 1979) and is the most abundant nuclear protein in *Xenopus* oocytes (Mills et al., 1980).

Stein et al. (1979) have shown that histone octamers can be stabilized at physiological ionic strengths by an equal weight of poly(glutamic acid) and that these octamers can be transferred to SV40 DNA in the presence of a "DNA-relaxing extract", or to short DNA (145 bp) by simple mixing. Stein (1979) has further shown that nucleosomes can bind additional histones as octamers in 0.6 M NaCl and can transfer these octamers to protein-free DNA. Ruiz-Currillo et al. (1979) demonstrated that chromatin assembly occurred at low salt concentrations in the absence of any assembly factors if mixing of DNA and histones was sufficiently slow (20–40% total weight of histones/h).

Germond et al. (1979) showed that chromatin extracts which were rich in topoisomerase I activity could also mediate chromatin assembly in vitro at physiological ionic strengths. Assembly could be mediated also by a highly purified topoisomerase I preparation, when used in stoichiometric amounts (equal weight to histones). In contrast to most of the previously cited studies, which described assembly factors interacting strongly with histones, this work suggested that assembly could be facilitated by a factor interacting primarily with DNA.

In a previous study (Nelson et al., 1979), we showed that extracts of *Drosophila* early embryos could mediate assembly of exogenous DNA and histones in vitro. Although these embryos contain a large pool of free maternal histones (R. Wiegand, unpublished experiments), we could find no abundant acidic histone binding protein analogous to that in the *Xenopus* oocyte system cited above (Laskey et al., 1977). The *Drosophila* extracts contained sufficient amounts of topoisomerase activities and histones to permit measurement of chromatin assembly by following the induction of negative supertwists (Germond et al., 1975) in relaxed plasmid DNA added to the extracts in the absence of either exogenous relaxing activity or exogenous histones. The work of Germond et al. (1979) suggested that the rapid chromatin assembly activity detected in *Drosophila* embryo extracts might be due entirely to the large amounts of topoisomerase I present in such extracts (Baase & Wang, 1974).

In this study, we show that a partially purified preparation of topoisomerase I from *Drosophila* embryos has no associated chromatin assembly activity. Further, we isolate an anionic assembly factor which accounts quantitatively for the activity of the *Drosophila* extracts and show that this factor is RNA. We show that RNA from many sources, including certain homopolymers, can mediate rapid chromatin assembly in vitro at physiological ionic strengths, provided the RNA is of sufficient length. The RNA acts stoichiometrically, being required in amounts greater than or equal to the amount of histone (by weight) present in the mixture, regardless of the

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[1] Abbreviations used: SV40, simian virus 40; EDTA, disodium ethylenediaminetetraacetate; DTT, dithiothreitol; $NaDodSO_4$, sodium dodecyl sulfate; HAP, hydroxylapatite; bp, base pairs; DEPC, diethyl pyrocarbonate; NEM, *N*-ethylmaleimide; HMG, high-mobility group.

amount of DNA present. Since the RNA assembly factor can be removed by ribonuclease treatment, this technique should prove useful for the large-scale assembly of homogeneous chromatins which are free of assembly factors. We also show that in the presence of RNA, chromatin-like structures can be assembled in vitro from DNA and histone mixtures containing only the arginine-rich histones, H3 and H4, or histone H4 alone. These results suggest a general mechanism for rapid assembly of chromatin in vitro, according to which histones are transferred from a polyanion (e.g., acidic protein or polypeptide, RNA, or chromatin) to DNA, thus avoiding the direct ionic interaction of DNA with histone which leads to nonspecific aggregates and precipitation at low ionic strengths.

Materials and Methods

Preparation of DNA, RNA, and Histones. Supercoiled pBR322 DNA was prepared as described previously (Nelson et al., 1979), except CsCl–ethidium bromide density gradients were centrifuged first in sealed tubes at 45 000 rpm for 24 h at 15 °C in the Beckman VTi50 vertical rotor and the plasmid band was directly recentrifuged in a Beckman SW50.1Ti rotor for 48 h at 37 000 rpm. Covalently closed, relaxed pBR322 DNA was prepared by treatment of supercoiled pBR322 DNA with the purified topoisomerase described below or with a chromatin extract from *Drosophila* Kc cells prepared according to Germond et al. (1979), followed by reisolation of covalently closed molecules by ethidium bromide–CsCl centrifugation in the SW50.1Ti rotor as described above. Preparations were relaxed at 4 °C to generate a distribution of species which could be resolved from nicked molecules of the same size in agarose gels at room temperature (e.g., see Figure 2).

Cytoplasmic RNAs from *Drosophila* Kc cells and *S. cerevisiae* were the gifts of Neil Osheroff and Tom St. John, respectively. Deoxyribonucleotide and ribonucleotide homopolymer preparations were obtained from P-L Biochemicals, Inc. Single-stranded phiX174 DNA was the gift of Joan Kobori. HMG fractions 1 and 17 were the gift of James Wang.

Histones were repurified from commercially extracted calf thymus histones (Sigma, type IIs) by the method of Bohm et al. (1973). Histones H1, H4, and H2B were isolated by Bio-Gel P60 gel filtration in 50 mM NaCl, and individual fractions of H2A and H3 were isolated from the unresolved mixture by gel filtration on Sephadex G100. Histone peaks from gel filtration were located by the absorbance at 230 nm, and pools of individual histones were rechromatographed on either Bio-Gel P60 (for H4 and H2B) or Sephadex G100 (for H3 and H2A) before being lyophilized and stored at −20 °C. *Drosophila* Kc cell salt-extracted histones prepared according to Germond et al. (1976) behaved identically with the calf histone preparations which were used for most experiments.

Topoisomerase Assay and Purification. Topoisomerase I (ATP independent) was partially purified from *Drosophila* embryo extracts as described by Hsieh & Brutlag (1980). A 2-mg sample of the hydroxylapatite-purified fraction (67 000 units/mg) was purified further by dialyzing into 0.1 M potassium phosphate, pH 7.0, 0.1 mM EDTA, 0.1 mM DTT, and 10% glycerol and loading on a 0.75 × 9 cm carboxymethyl-Sephadex (CM-50) column. The column was washed with 30 mL of the same buffer and eluted with a 150-mL linear gradient from 0.1 to 0.6 M potassium phosphate, pH 7.0, in 0.1 mM EDTA, 0.1 mM DTT, and 10% glycerol. Topoisomerase activity eluted between 0.3 and 0.4 M potassium phosphate. Pooled fractions were concentrated by step elution with 0.8 M potassium phosphate from a 1-mL hydroxylapatite column. After addition of glycerol to 50%, the topoisomerase could be stored at −20 °C for at least 6 months without loss of activity. Overall recovery of topoisomerase activity from extracts was approximately 5%, but an ATP-dependent topoisomerase (type II) which is eluted from the phosphocellulose column at 0.4 M NaCl is responsible for 50% of the DNA relaxing activity in crude extracts when assayed in the presence of magnesium (Hsieh & Brutlag, 1980).

Topoisomerase I prepared according to the above procedure was not measurably contaminated with nucleic acids and had no detectable endonuclease activities. The enzyme was dependent upon addition of magnesium only if dialyzed extensively against EDTA. Nondialyzed HAP–topoisomerase and CM-Sephadex–topoisomerase fractions were not magnesium dependent and were equally active in the chromatin assembly assay described below.

Topoisomerases were assayed at 30 °C as described by Hsieh & Brutlag (1980), except that topoisomerase fractions during purification were assayed in the presence of 10 mM Tris-HCl, pH 7.6, and 10 mM $MgCl_2$ to lessen the effect of differing ionic strengths. Resolution of pBR322 DNAs of differing superhelical densities was by electrophoresis in horizontal 1% agarose slab gels in 36 mM Tris, 30 mM NaH_2PO_4, and 1 mM EDTA, pH 7.7, until the xylene cyanol FF marker dye migrated one-half to two-thirds the length of the gel. Gels were stained with ethidium bromide and photographed with short-wavelength UV light. One unit of topoisomerase is defined as the amount of enzyme that can relax 0.3 μg of DNA under standard assay conditions (no magnesium, 150 mM NaCl).

Chromatin Assembly Assay. Chromatin assembly was routinely measured by incubation for 45 min at 30 °C of 20-μL reactions containing 0.3 μg of relaxed covalently closed pBR322 DNA, 0.3 μg of calf thymus core histones, a variable amount of embryo extract or assembly factor, and 2 units of purified topoisomerase I in 20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.01% NP-40, 1 mM EDTA, and 10% glycerol. Reactions were stopped by the addition of 3 μL of 5% NaDodSO$_4$, 0.25 M EDTA, 50% glycerol, 0.05% bromphenol blue, and 0.05% xylene cyanol FF, and the superhelical state of the DNA was analyzed by electrophoresis in 1% agarose slab gels as described for topoisomerase assays.

Preparation of Drosophila Embryo Extracts. Extracts were prepared from 1-h postfertilization *Drosophila* embryos as described previously (Nelson et al., 1979). DEAE-cellulose eluate was prepared by passage of the extract through 2 volumes of DEAE-cellulose (Whatman DE-52) in 0.2 M potassium phosphate, pH 7.0, 0.1 mM EDTA, 0.1 mM DTT, and 10% glycerol, washing with 4 volumes of the same buffer, and eluting with 2 volumes of the same buffer plus 1 M NaCl. Macromolecules in the eluate were precipitated by addition of ammonium sulfate to 70% and resuspended in the original volume of 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.01% NP-40, and 10% glycerol.

Quantification of Protein, DNA, and RNA. Protein was measured by the method of Lowry et al. (1951), as modified by Bensadoun & Weinstein (1976). Samples were always precipitated with 10% trichloroacetic acid before performing protein analysis. Standard curves were prepared by using bovine serum albumin and histone stock solutions of known concentration. Histone standards were consistently 21% higher than serum albumin standards in the colorimetric assay.

pBR322 DNA from nucleoprotein complexes was measured by integration of band areas on Polaroid 665 negatives of ethidium fluorescence patterns from 1% agarose gels on which

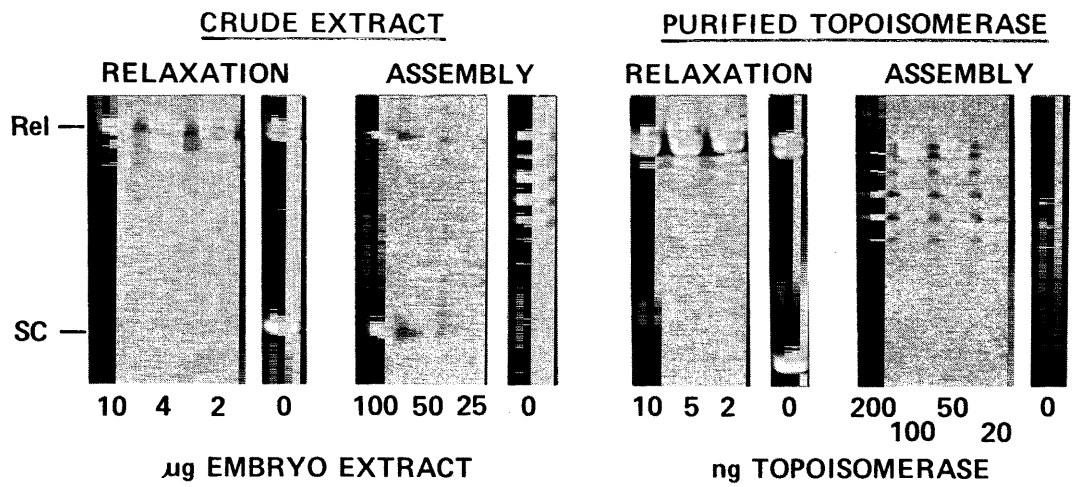

FIGURE 1: Topoisomerase I from *Drosophila* embryos lacks chromatin assembly activity. In lanes marked "relaxation", the indicated amounts of *Drosophila* embryo extract or purified topoisomerase I (CM-Sephadex fraction) from the same source were used to treat 0.3 μg of supercoiled pBR322 DNA for 30 min under the relaxation reaction conditions described under Materials and Methods. In lanes marked "assembly", the indicated amounts of extract or purified enzyme were used in assembly reactions with 0.3 μg of relaxed pBR322 DNA and 0.3 μg of calf thymus core histones, but without additional assembly factors. "SC" and "Rel" indicate the positions at which fully supertwisted and fully relaxed pBR322 DNA migrated.

unknowns and dilutions of standard DNA stocks were electrophoresed as described above. Bands were traced and their relative areas integrated by using a Helena Laboratories Quickscan Jr. microdensitometer equipped with a variable speed motor. DNA unknowns and standard dilutions were adjusted to fall within the linear portion of the Polaroid film exposure vs. density curve. Purified nucleic acid samples were quantified by measuring the absorbance at 260 nm by using extinction coefficients of 20 and 25 for 1 mg/mL solutions of DNA and RNA, respectively. Extinction coefficients used for 1 mg/mL solutions of homopolymers were as follows: poly(rA), 21.6; poly(rU), 23.1; poly(rC), 14.3; poly(dA), 28.4 (at 264 nm); poly(dC), 25.0 (at 274 nm); poly(rI)–poly(rC), 25.0.

Results

Topoisomerase I from Drosophila Embryos Lacks Chromatin Assembly Activity. Crude extracts from *Drosophila* embryos prepared as described previously (Nelson et al., 1979) can mediate both the relaxation of supertwisted DNA and its assembly into chromatin in the presence of histones. To determine if a topoisomerase in the extract was responsible for both activities, as suggested by the work of Germond et al. (1979), we compared the relative amounts of relaxing and assembling activities of the crude extract with those of a type I topoisomerase which was extensively purified from the same extracts as described under Materials and Methods. Figure 1 shows that chromatin assembly in vitro at 0.15 M NaCl required 25 times more of the crude extract (100 μg/0.3 μg of DNA) than did complete relaxation of the same amount of DNA (4 μg/0.3 μg of DNA). In contrast, the purified topoisomerase was incapable of chromatin assembly, even at levels 200 times that amount required to completely relax the same amount of DNA. The topoisomerase activity of the extracts was separated from the chromatin assembly activity in the first step of purification (Polymin-P precipitation). A second, ATP-dependent (type II) topoisomerase has been purified from the same embryo extracts (Hsieh & Brutlag, 1980) and is similarly devoid of any intrinsic chromatin assembly activity (data not shown). Changes in order of addition of reaction components, stepwise addition of components, and preincubation of combinations of reaction components all gave the same negative results. Topoisomerase activity remained in incubations at 30 °C for at least 2 h (data not shown),

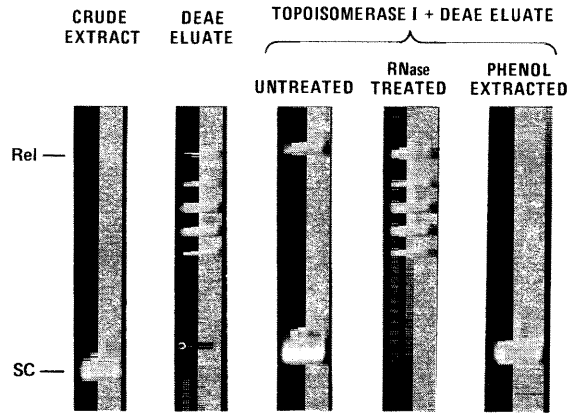

FIGURE 2: DEAE-cellulose separates a chromatin assembly factor from topoisomerase activity. Relaxed pBR322 DNA was incubated in standard assembly reactions in the presence of 5 μL of crude embryo extract (track 1), 5 μL of DEAE-cellulose and 1 M NaCl eluate prepared as described under Materials and Methods (track 2), 5 μL of eluate plus 2 units of purified topoisomerase I (track 3), 5 μL of eluate which was treated with 0.1 unit of pancreatic ribonuclease for 10 min at 22 °C followed by 0.1% DEPC before addition of topoisomerase (track 4), or 5 μL of eluate which was phenol extracted, ethanol precipitated, and resuspended in the original volume, plus 2 units of topoisomerase.

suggesting that rapid loss of relaxation activity upon dilution was not responsible for the lack of detectable assembly activity.

DEAE-cellulose Separates a Chromatin Assembly Factor from Topoisomerase Activity. In the previous experiment, chromatin assembly activity was eliminated from a crude topoisomerase preparation by fractionation with Polymin-P. The activity could not be recovered by high salt elution of the Polymin-P pellet, so an alternative method, DEAE-cellulose chromatography, was employed for removal of nucleic acids (Jovin et al., 1969). Figure 2 shows that under these conditions we obtained two fractions; one (the DEAE-cellulose pass-through) contained all of the topoisomerase activity and no chromatin assembly activity, and the second (eluted from DEAE-cellulose with 1 M NaCl) contained a factor which mediated chromatin assembly in the presence of catalytic amounts of topoisomerase I. This factor would facilitate chromatin assembly in the presence of either the DEAE-cellulose pass-through or the highly purified topoisomerase described under Materials and Methods. Figure 2 also shows

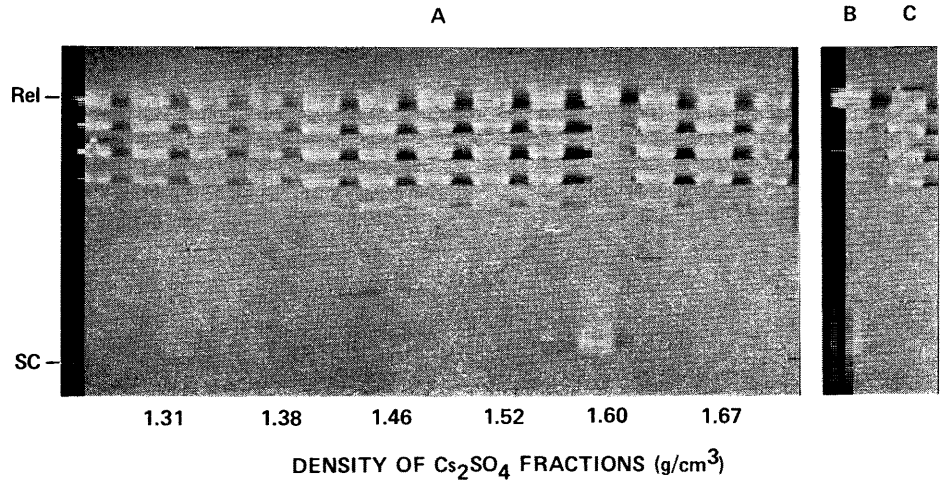

FIGURE 3: Assembly factor in embryo extract is RNA. A 250-μL sample of the DEAE-cellulose eluate was adjusted to a density of 1.50 g/cm^3 and centrifuged for 48 h at 38 000 rpm in the SW50·1Ti rotor. Fractions were analyzed for density by refractometry and dialyzed against 20 mM Tris, pH 7.5, and 1 mM EDTA to remove Cs_2SO_4. In tracks marked "A", 5-μL aliquots from fractions with the indicated densities were assayed in assembly reactions as described under Materials and Methods. Track B shows the activity of 1 μL of the eluate before Cs_2SO_4 centrifugation, and track C shows an assembly reaction with topoisomerase I only (no other factors).

that the factor eluted from DEAE-cellulose was destroyed by brief treatment with protease-free pancreatic ribonuclease. In control experiments, addition of pancreatic ribonuclease which was first inactivated by DEPC did not inhibit the chromatin assembly assay, nor did DEPC pretreatment of the DEAE-cellulose eluate (data not shown). The assembly factor could be recovered quantitatively from the aqueous phase of a phenol extraction of the DEAE-cellulose eluate (Figure 2). These two experiments strongly suggest that the assembly factor from the extracts is not a protein, although they do not rule out the possibility that a covalent protein–nucleic acid association is responsible.

Assembly Factor in Embryo Extract Is RNA. To determine whether the assembly factor was protein, DNA, RNA, or some hybrid of these, we centrifuged a sample of the active DEAE-cellulose eluate to equilibrium in a Cs_2SO_4 density gradient in the presence of the detergent Sarkosyl and assayed various density fractions following removal of Cs_2SO_4. As shown in Figure 3, the assembly activity was recovered from a fraction of density 1.6 g/cm^3, the same density at which a pure RNA marker banded in a parallel gradient (data not shown). Pure DNA reaches equilibrium in such gradients at a density of about 1.4 g/cm^3, while protein floats. The activity recovered at density 1.6 g/cm^3 was quantified by assaying dilutions and accounted for at least 80% of the activity initially present (data not shown). Since the DEAE-cellulose eluate accounts for nearly all of the assembly activity detected in crude extracts, these results along with those in Figure 2 indicate that the most abundant assembly factor in *Drosophila* embryo extracts is RNA.

Exogenous RNAs Can Complement Topoisomerase for Chromatin Assembly. In order to test whether assembly was due to an RNA species unique to the embryo extracts or was a general property of any RNA, we assayed exogenous natural and synthetic RNA preparations, as well as a variety of other polyanions, by the supertwisting assay used above. Table I shows that a variety of RNA species act as chromatin assembly factors, with varying efficiencies. Relative activities are expressed as the amount by weight of a given preparation which can completely supertwist 1 μg of circular covalently closed pBR322 DNA in the presence of 1 μg of calf thymus core histones. Preparations of total cytoplasmic RNA from *Drosophila* embryos, *Drosophila* Kc cells, and wild-type *S. cerevisiae* were all active in assembly in vitro. Large amounts of each preparation were required relative to the amount of histones and DNA being assembled, unless the fraction of RNA larger than 250 bases was selected by sucrose-gradient fractionation (see Table II), in which case assembly was efficient with an equal weight of RNA. The shorter fraction of RNA was completely inactive, as were preparations of purified yeast tRNAs.

Table I: Efficiency of Natural and Synthetic Polyanions as Chromatin Assembly Factors[a]

assembly factor	μg of DNA assembled/100 μg of factor
embryo extract RNA	7
Kc cell cytoplasmic RNA	13
yeast cytoplasmic RNA, unfractionated	6
yeast cytoplasmic RNA, over 250 bases	100
poly(rU)	3
poly(rA)	4
poly(rC)	0
poly(dT), -(dA), -(dC)	0
poly(rI-rC) (ds)	0
ss phiX-174 DNA	0
ds salmon sperm DNA	0
ss salmon sperm DNA (heat denatured)	0
yeast tRNA	0
poly(glutamic acid)	100
HMG proteins, fractions 1 and 17	0
heparin sulfate	0

[a] Chromatin assembly reactions were performed as described under Materials and Methods by using 0.3 μg of relaxed pBR322 DNA, 0.3 μg of calf thymus histones, a variable amount of assembly factor, and 2 units of topoisomerase I. Assembly factors were titrated over a range of 0.05–10.0 μg per reaction, and the numbers presented represent the ratio of DNA assembled to factor in the region of half-maximal assembly as measured by the supercoiling assay. All assembly factors were premixed with histones before exposure to DNA and topoisomerase. In some cases, consecutive titration points differed by 2-fold, and, therefore, relative values are only accurate within a factor of 2. Polyanions lacking activity were tested separately in relaxation reactions to ensure they did not inhibit topoisomerase activity at the levels tested.

Certain homopolymer preparations of RNA were also active (Table I). Poly(rU) and poly(rA) both mediate chromatin assembly, when used in 10–20 times weight excess over histones and DNA. Size fractionation of these homopolymer preparations only slightly increased their activity (data not shown). Poly(rC) was completely inactive, even when tested

Table II: Dependence of Chromatin Assembly on RNA Length[a]

average length (bases)	μg of DNA assembled/100 μg of RNA
5300	60
3500	50
2400	100
1500	85
660	80
240	0
57	0
starting material	6

[a] Total RNA from *S. cerevisiae* was fractionated in 10–30% sucrose gradients in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl, and 0.1% DEPC. Fractions were concentrated by ethanol precipitation and resuspended in the same buffer without DEPC before being tested as assembly factors in standard assembly reactions. The size of the RNA in each fraction was measured by electrophoresis of glyoxylated RNA samples (McMaster & Carmichael, 1977).

in great excess over the other assembly reaction components. Similarly, none of the deoxyhomopolymers tested [poly(dA), poly(dT), and poly(dC)] exhibited any assembly activity, nor did a preparation of double-stranded poly(rI)–poly(rC). Deoxypolymers inhibited the activity of poly(rU) and poly(rA) if present in the same mixture. Such inhibition occurred in an approximately competitive manner, the addition of an equal weight of poly(dA) reducing by approximately 50% the assembly activity of poly(rA).

Several other polyanions were also tested. Natural single- and double-stranded DNAs and heparin sulfate (a polysaccharide) were inactive. Of particular interest is the observation that neither of the two preparations of HMG proteins tested was active in assembly, although this class of proteins is similar in amino acid composition to the protein purified by Laskey et al. (1979) as an assembly factor. As previously reported by Stein et al. (1979), poly(glutamic acid) is active at an equal weight to histones and DNA. In the case of each active preparation, it was necessary to mix the histones first with the polyanion before adding DNA and topoisomerase for the observation of maximal activity.

Chromatin-like Structures Can Be Formed from H3 and H4 or from H4 Alone. In an effort to define the substrate requirements of the in vitro assembly system, we included the arginine-rich histones H3 and H4 or histone H4 alone in assembly reactions with approximately equal weights of poly(rU). As shown in Figure 4, either of these subsets of the four core histones fully supertwisted relaxed pBR322 DNA as well as the full complement of core histones. None of the other subsets tested (H3, H2A, or H2B alone; H2A and H2B, H4 and H2A, H4 and H2B) could supertwist DNA under the same conditions (data not shown), nor could polyamines, protamines, polylysine, or polyarginine when tested over wide ranges of concentration (0.1–20 times the weight of DNA).

Complete assembly of complexes containing H3 and H4 or H4 alone requires a higher concentration of histones than is optimal when all four core histones are present (Figure 4). H3- and H4-containing complexes were fully twisted when the histones were present at 75 μg/mL; 150 μg/mL H4 must be present for complete twisting of DNA. Although there was a 10-fold excess of histone H4 over DNA in these reactions, the H4 preparation used was at least 99% pure as judged by the presence of a single band in heavily overloaded 18% Na-DodSO$_4$–acrylamide gels (Figure 5c) and acid–urea–acrylamide gels (Laemmli, 1970; Panyim & Chalkley, 1969). No H3 contamination was detectable by Coomassie blue staining

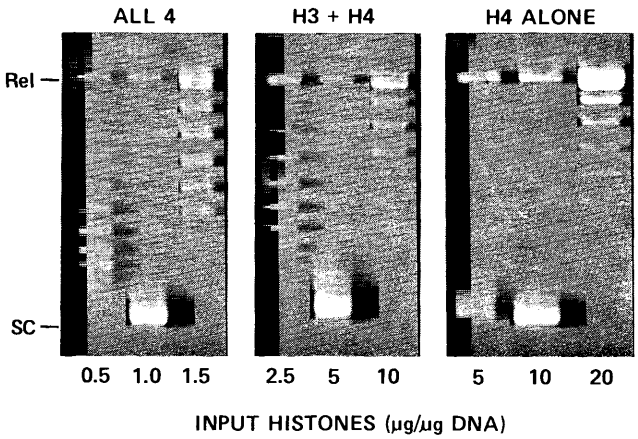

FIGURE 4: Chromatin-like structures can be formed with histones H3 and H4 or H4 alone. Assembly reactions were performed as described under Materials and Methods, except histones were present at the indicated levels. Total histone concentrations were 7.5, 15.0, and 22.5 μg/mL for reactions with all four histones, 37.5, 75, and 150 μg/mL for H3 and H4, and 75, 150, and 300 μg/mL for H4 alone. A 9-μg sample of poly(rU) was included in each reaction as an assembly factor.

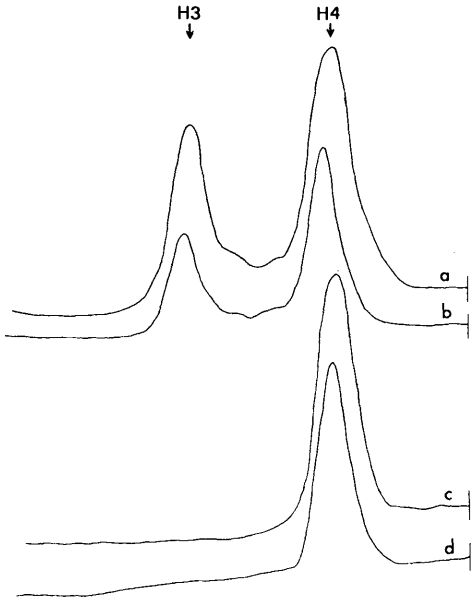

FIGURE 5: NaDodSO$_4$–acrylamide electrophoresis of histone fractions H3–H4 and H4 used for assembly. Histones prepared as described under Materials and Methods were electrophoresed in 18% NaDodSO$_4$–polyacrylamide gels, stained with Coomassie blue stain, and photographed on Polaroid 665 film with transmitted light. The negative was scanned with a Helena Laboratories Quickscan Jr. microdensitometer. Traces a and c show the histone fractions used for assembly in Figures 4 and 6 while traces b and d show the histones recovered from the nucleoprotein complexes purified from the excess histones by gel filtration on Bio-Gel A1.5m.

of either gel type. In addition, histones recovered from the nucleoprotein complexes formed from either H3 and H4 or H4 alone are identical with the input histones and show no enrichment for any contaminating histone species (Figure 5b,d).

The concentration of histones required for the supertwisting of a fixed level of DNA is not reflected in the composition of the final product. Nucleoprotein complexes were assembled from relaxed pBR322 DNA in the presence of H4 alone, H3 and H4, or all four core histones. Reactions were carried to completion as judged by the extent of supertwisting, and the complexes were treated with ribonuclease, isolated by gel filtration, and subjected to protein and DNA analysis, all as described under Materials and Methods. The three types of

complexes assembled, with all four core histones present, H3 and H4 present, and H4 alone present, had final ratios of histone to DNA of 1.1, 3.1, and 0.9, in contrast to their respective input ratios of histones to DNA of 1.0, 5.0, and 10.0. Aliquots of the above reactions were analyzed on 18% Na-DodSO$_4$–acrylamide gels and contained histones in proportions identical with the original reaction mixtures. Complexes with core histones contained equimolar amounts of H2A, H2B, H3, and H4 (data not shown) while complexes with H3 and H4 or H4 alone contained only equimolar H3 and H4 or H4 alone (Figure 5b,d).

To determine whether assembly of nucleoprotein complexes of each kind resulted in DNAs with physiological superhelical densities, we subjected DNA from samples of each species to electrophoresis in the presence or absence of 10 μg/mL chloroquine, as described under Materials and Methods, and counted bands by the method of Shure et al. (1977). The DNA from the most completely assembled complexes in each of the three cases contained 19–21 negative supertwists, a figure consistent with that found in natural minichromosomes of this size (4362 bp). Thus, fully assembled complexes of each type had DNAs with chromatin-like superhelical densities, rather than detectably lower [as in the case of HU protein–DNA complexes (Rouviere-Yaniv et al., 1979)] or higher numbers [as in the case of PM2 bacteriophage DNA (Shure et al., 1977)].

Micrococcal Nuclease Digestions of Nucleoprotein Complexes Assembled in Vitro. To further characterize the nucleoprotein complexes containing pBR322 DNA and the three histone combinations described above, we digested complexes which were assembled and reisolated as described for Figure 4 with increasing amounts of micrococcal nuclease at 22 °C, and the DNA products were electrophoresed as described under Materials and Methods. Figure 6 shows that the DNA in all three complexes was first degraded to a continuum of sizes larger than 160 bp, with a fraction of the DNA present as fragments which are multiples of approximately 160–170 bp (Figure 6d,f,h). A second class of discrete DNA fragments is produced in digestions of all three nucleoprotein complexes which appear to be multiples of 130–140 bp. The bulk of the DNA larger than 160 bp is not present in discrete fragments. The presence of discrete fragments which are multiples of 130–140 or 160–170 bp in the digestions of each of the nucleoprotein complexes suggests that that fraction of the DNA was present in closely packed nucleoprotein particles, each protecting about 140 or about 160 bp of DNA. The presence of two apparently independent repeats suggests that formation of the 140- or 160-bp particle may be cooperative phenomena, with particles of the same size most likely to be adjacent to one another. Digestion of unassembled mixtures of pBR322 DNA with and without the RNA assembly factor present produced no discrete fragments, nor did mixtures of assembly factor and DNA (data not shown).

Increased digestion of the assembled complexes (Figure 6e,g,i) with micrococcal nuclease reduced the size of DNA fragments to approximately 140 bp and a series of smaller fragments ranging from 140 to about 50 bp. Complexes containing all four core histones were reproducibly digested to a series of 8–10 fragments smaller than 160 bp and which coelectrophoresed with fragments from similar digestions of native chromatin (Figure 6b,c). Digestions of complexes containing H3 and H4 or H4 alone produced a subset of the native fragments which lacked four to five of the larger fragments (data not shown), suggesting that the complexes shared some, but not all, of the structural features of complexes

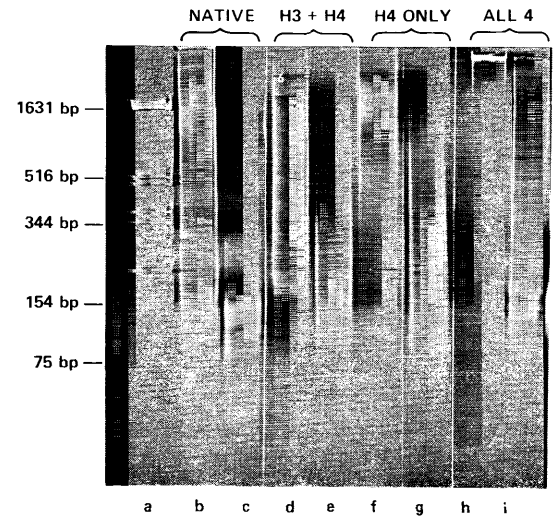

FIGURE 6: Micrococcal nuclease digestions of chromatin-like complexes containing core histones, H3 and H4, or H4 alone. Nucleoprotein complexes were assembled with the indicated histone combinations by using poly(rU) as the assembly factor in the presence of 2 units of purified topoisomerase I per μg of DNA. The complexes were passed through Bio-Rad A1.5m columns to remove free histones and assembly factors and treated with micrococcal nuclease. The DNA products were phenol extracted, washed twice with ethyl ether, and ethanol precipitated before loading on a 5% acrylamide gel which was prepared and run as described by Maniatis et al. (1975). Track a shows size markers generated by *Hin*fI restriction of pBR322 DNA. Tracks b and c show DNA from the digestion of *Drosophila* Kc cell nuclei containing 5 μg of DNA with 200 units/mL micrococcal nuclease for 30 s (b) or 15 min (c). Tracks d–i show DNA from the digestion of nucleoprotein complexes (5 μg each) assembled with H3 and H4 (d and e), H4 alone (f and g), or all four core histones (h and i) as described under Materials and Methods. Samples were treated for 5 min at 20 °C with either 50 units/mL (d, f, and h) or 200 units/mL (e, g, and i) micrococcal nuclease.

with all four core histones. Nucleoprotein complexes with H3 and H4 or H4 alone were both slightly more sensitive to digestion than complexes with all the core histones.

Discussion

Our results show that the purified topoisomerase I from *Drosophila* embryos does not have an intrinsic chromatin assembly activity. Rather, the ability of *Drosophila* extracts to mediate chromatin assembly in vitro at physiological ionic strengths resides in a highly anionic fraction of the extracts which we demonstrate to be largely RNA. Exogenous natural and homopolymer RNAs, if of sufficient length, can also mediate chromatin assembly in vitro. The RNA acts stoichiometrically in assembly, being required in at least equal weight to the amount of histones present in the reaction mixture. Natural and homopolymer DNAs, both single and double stranded, are inactive under the same conditions. We have also shown that the arginine-rich histones H3 and H4 or histone H4 alone is suficient to produce nucleoprotein complexes with physiological numbers of supertwists in the DNA. The complexes containing these subsets of the four core histones resembled assembled complexes containing all four histones with respect to some patterns of nuclease sensitivity, although complexes containing all four core histones more closely resembled native chromatin in nuclease digestions.

The results of Germond et al. (1979) suggest that large amounts of a type I topoisomerase, present either as a chromatin extract or as a highly purified enzyme, might be sufficient to assemble chromatin. The enzyme was proposed to act both stoichiometrically, as an assembly factor, and enzymatically, to relieve topological constrainsts in covalently closed

or otherwise constrained DNA substrates. We have found (data not shown) that the assembly activity in such chromatin extracts is ribonuclease sensitive. In addition, the aqueous phase of phenol extractions of such "relaxing extracts" can complement enzymatic amounts of our purified topoisomerase I preparation in chromatin assembly. This fraction quantitatively accounts for the assembly activity in the extract. Such "relaxing extracts" should thus be used with caution in the characterization of other assembly factors because of the possibility of contamination with RNA. We have not performed similar experiments with the purified topoisomerase described by Germond et al. (1979). The trypsin and NEM sensitivities of the chromatin assembly observed in crude extracts probably reflect inactivation of the DNA topoisomerase I (Nelson et al., 1979). The purified *Drosophila* DNA topoisomerase I is completely inhibited by 10 mM NEM (data not shown).

In four separate preparations of the topoisomerase I (ATP independent) from *Drosophila* embryos, the chromatin assembly activity in the extract was separated from the topoisomerase in the first step of purification (removal of nucleic acids by Polymin-P precipitation or DEAE-cellulose chromatography). It is possible, but unlikely, that we have isolated a fragment of the topoisomerase which lacks a normally associated assembly activity. Recently, L. Liu and J. Wang, K. Javaharian (unpublished experiments) have isolated type I topoisomerases from HeLa cells and *Drosophila* embryos, respectively, which are present as larger complexes (~120 000 daltons) than the nicking–closing enzymes (~68 000 daltons) previously reported from HeLa cells (Keller, 1975) and rat liver (Champoux & McConaughy, 1976). The larger complexes are magnesium dependent and can be converted to the smaller form by limited proteolysis. Since our topoisomerase preparations are magnesium dependent after dialysis against EDTA (see Materials and Methods), it is likely that we have isolated the larger, intact form of the enzyme. As shown under Results, even a 200-fold excess of this topoisomerase over that needed to relax a given amount of DNA will not by itself mediate the assembly of the same DNA.

All of our experiments made use of covalently closed plasmid DNA and required a topoisomerase for the relief of topological constraints. Thus, they do not address the question of whether or not a topoisomerase is required during the assembly of chromatin from nicked circular or linear DNAs, in which such topological constraints might not exist. Experiments in which the topoisomerase was present during the assembly incubation or was not added until the end (data not shown) suggest that the enzyme must be present during the course of incubations with covalently closed DNAs. This may indicate that DNA must be maintained in a relaxed state for assembly to be favorable. If this is the case, it is likely that any topoisomerase treatment or other procedure capable of relaxing the positive supertwists generated by assembly of the relaxed DNA substrate should be able to substitute for the type I topoisomerase used in these experiments. However, we have thus far been unable to find conditions under which the type II topoisomerase from *Drosophila* (Hsieh & Brutlag, 1980) can relax nucleoprotein complexes for measurement of chromatin assembly by the supercoiling assay. Assembly reactions monitored by treatment with micrococcal nuclease suggest that a topoisomerase must be present for efficient assembly of chromatin even from nicked circular pBR322 DNA (data not shown).

Our results show that long RNAs are among a number of macromolecules able to mediate efficient and rapid chromatin assembly in vitro at low ionic strength. This enables us to propose a model incorporating the common features of similar "assembly factors" described thus far. The majority of materials which have been reported to act as assembly factors are macromolecules containing a fixed distribution of negative charges [e.g., an acidic protein (Laskey et al., 1978; Baldi et al., 1978), an acidic polypeptide (Stein et al., 1979), or chromatin (Stein, 1979)]. Assembly factors appear to both stabilize the correct histone–histone associations in low salt concentrations and mediate the transfer of correctly associated histones to strongly binding DNA. As shown by Stein et al. (1979), anionic macromolecules are able to stabilize histone octamers at physiological ionic strength. The role of assembly factors in mediating histone–DNA interactions appears to be at least partially one of masking unassociated histones from rapid association with DNA. Although Ruiz-Carrillo et al. (1979) have shown that histones and DNA mixed sufficiently slowly without a mediating factor can produce a chromatin product, we never observe low-salt assembly of long DNA (greater than 1 kilobase) into chromatin in the absence of some anionic factor. Rather, unmediated histone–DNA mixtures precipitate in complexes which exhibit none of the features of chromatin.

It is likely that the interactions of some polyanions with histones are sufficient to stabilize histone octamers yet are too strong to permit transfer of the octamers to double-stranded DNA for chromatin assembly. This stronger competition for histone binding might explain why double- and single-stranded DNAs and certain RNAs are unable to act as assembly factors. It has recently been reported that single-stranded DNA, but not RNA, can form stable complexes with histones that resemble nucleosomes containing double-stranded DNA (Palter & Alberts, 1979; Palter et al., 1979). A more detailed study of the effectiveness of various anions as assembly factors and of histone binding to these anions should reveal more about the charge distributions and relative affinities that permit octamer formation at low ionic strengths.

Our results and those of Stein et al. (1979), Laskey et al. (1978), and Baldi et al. (1978) imply that histone–DNA interactions in vivo must be subject to competition and interaction with anionic macromolecules such as RNA, acidic proteins, and chromatin. Cells may have a variety of mechanisms and adaptations for mediating histone–DNA interactions under conditions of histone excess (as in some oocytes and early embryos) or histone equivalence (as in somatic cells). It is possible, as suggested by Stein et al. (1979) and Ruiz-Carrillo et al. (1979), that the presence of large amounts of assembly factors is crucial in cells with pools of free histones, while cells with lower steady-state levels of histones in their nuclei can undertake assembly of histones into chromatin without other factors (Ruiz-Carrillo et al., 1979) or by utilizing chromatin itself as a mediating factor (Stein, 1979). The large number of polyanions which are active in vitro as chromatin assembly factors makes it difficult to conclude which of these, if any, is the responsible factor in vivo.

It is of interest that nucleoprotein complexes containing only the arginine-rich histones H3 and H4 or histone H4 alone could be assembled which resemble complexes with all four core histones. Previous reports showed that the histone pair of H3 and H4 could act to supertwist circular DNA (Bina-Stein & Simpson, 1977; Camerini-Otero & Felsenfeld, 1977) and could generate chromatin-like patterns of micrococcal nuclease sensitivity (Camerini-Otero et al., 1976; Jorcano & Ruiz-Carrillo, 1979; Stockley & Thomas, 1979) in high salt reconstitution experiments. Ruiz-Carrillo and Jorcano further

demonstrated that H3–H4 tetramers can organize nucleosome formation if prebound to DNA and followed by introduction of H2A and H2B (Jorcano & Ruiz-Carrillo, 1979; Ruiz-Carrillo et al., 1979). The high ratio of histone to DNA that we find in these H3- and H4–DNA complexes (3:1) is reproducible and may represent the binding of excess H3–H4 tetramers to core particles as has been observed for histone octamers by Stein (1979).

Pure H4–DNA complexes which resemble chromatin have not been described previously, possibly because of the high concentration of H4 which is required during assembly. We find that H4 can form specific complexes if present at a concentration of 150–750 μg/mL (see Results) and that complexes resemble chromatin with respect to DNA supercoiling and nuclease sensitivity. The structure of these H4-containing complexes is not likely to be due to contaminating H3 (or H2A and H2B), because of the absence of other histones in the purified H4 preparation (see Materials and Methods) and because gel analysis of histones recovered from the complexes demonstrated the presence only of H4 (see Results). It is possible that the high concentration of H4 required reflects the generation of a histone H4 fiber or other structure which enables H4 to form a regular framework around which DNA can wind (Sperling & Bustin, 1975). Since histone H4 fibers exhibit diffraction patterns which resemble those of chromatin (Sperling & Amos, 1977), it is not surprising to find that such aggregates, if formed in our assays, can order the supertwisting of DNA and its protection from nucleases. The sufficiency of H3 and H4 or of H4 alone for the generation of several chromatin features in assemblies in vitro reaffirms observations of other workers that the arginine-rich histones are responsible for the largest part of the structural organization of the nucleosome [for a review, see McGhee & Felsenfeld (1980)].

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