Evidence for Fidelity of Chromatin Reconstitution†

G. S. Stein,* R. J. Mans, E. J. Gabbay, J. L. Stein, J. Davis, and P. D. Adawadkar

ABSTRACT: Several lines of evidence are presented which support the contention that chromatin may be dissociated, fractionated, and reconstituted without altering the compositional, structural, or transcriptional integrity of the genome. The similar compositions of native and reconstituted chromatins are suggested by the absence of significant differences in their protein/DNA ratios and in the polyacrylamide gel electrophoretic profiles of their histones and non-histone chromosomal proteins. Criteria for fidelity of genome structure in reconstituted chromatin include binding of reporter molecules with specificity for the minor groove of DNA, binding of histones, number of sites available for addition of nucleotides, and circular dichroism spectra. When the transcriptional activities of native and reconstituted chromatins were compared under conditions where reinitiation is prohibited, significant changes were not observed. Taken together, the present results strongly suggest, but do not conclusively establish, fidelity of chromatin reconstitution.

The genome of eukaryotic cells is a nucleoprotein complex, referred to as chromatin, consisting primarily of DNA and two classes of chromosomal proteins—histones and non-histone chromosomal proteins. Histones have been shown to be involved in repression of DNA-dependent RNA synthesis as well as in the structural organization of the genome (Allfrey et al., 1963; Hnilica et al., 1971; Huang and Bonner, 1965; Louie and Dixon, 1972; Kornberg and Thomas, 1974; Kornberg, 1974). In contrast, evidence is accumulating which suggests that among the non-histone chromosomal proteins are macromolecules which may recognize specific genetic loci and play a key role in the regulation of gene read-out (Wang, 1968; Kleinsmith et al., 1970; Gilmour and Paul, 1970; Spelsberg and Hnilica, 1970; Teng et al., 1971; Stein and Farber, 1972; Stein et al., 1972; Kostraba and Wang, 1973; Stein et al., 1974a). While much speculation concerning the control of transcription has arisen from correlating changes in gene expression with modifications in the proteins which comprise the genome and presumably regulate its function, the evidence is at best circumstantial. However, dissociation of chromatin into its constituent nucleic acid and protein components followed by reconstitution with selected classes of these macromolecules and assay of the RNAs transcribed from such reconstituted chromatin preparations has been an extremely powerful approach which has provided direct evidence for the regulation of transcription by chromosomal proteins (Gilmour and Paul, 1970; Spelsberg and Hnilica, 1970; Stein and Farber, 1972; Stein et al., 1972). Undoubtedly, this approach will be instrumental in further elucidating the mechanism by which chromosomal proteins control gene expression. It is therefore imperative to establish that reconstituted chromatin is a bona fide representation of chromatin as isolated from intact cells. Several lines of evidence already suggest fidelity of chromatin reconstitution (Gilmour and Paul, 1970; Stein and Farber, 1972; Stein et al., 1972; Paul and Gilmour, 1968; Paul and More, 1972; Bekhor et al., 1969; Roti Roti et al., 1974; Spelsberg and Hnilica, 1970; Huang and Bonner, 1965). In the present communication we present additional lines of evidence which support the contention that chromatin can be dissociated, fractionated, and reconstituted without significantly disrupting the integrity of the genome.

Materials and Methods

Preparation of Nuclei and Chromatin. A. HeLa S_3 CELLS. Nuclei were isolated from exponentially growing HeLa S_3 cells (Stein and Borun, 1972) and chromatin was prepared as previously described (Stein and Farber, 1972). All procedures were carried out at 4°. Cells were washed three times with 60 volumes of Earle's balanced salt solution and lysed in 80 volumes of 80 m*M* NaCl–20 m*M* EDTA–1% Triton X-100 (pH 7.2). Nuclei were pelleted by centrifugation at 1000*g* for 10 min. The nuclei were washed three times with 80 volumes of the lysing medium and then with 80 volumes of 0.15 *M* NaCl–0.01 *M* Tris (pH 8.0). Nuclei isolated utilizing this procedure are free of visible cytoplasmic contamination when examined by phase contrast and electron microscopy. Chromatin was prepared by lysing nuclei in 80 volumes of triple glass-distilled water with several gentle strokes of a wide clearance Teflon homogenizer. The chromatin was allowed to swell in an ice bath for 20 min, pelleted by centrifugation at 12,000*g* for 15 min, redispersed in distilled water by homogenization, and again pelleted by centrifugation at 12,000*g* for 15 min. These procedures result in minimal shearing of chromatin. The protein/DNA ratios of such chromatin preparations are 1.8.

B. RAT LIVER. Charles River male rats (average weight 400 g) were sacrificed by cervical decapitation. The livers were immediately dissected and homogenized in 0.25 *M* sucrose–0.05 *M* KCl–0.005 *M* $MgCl_2$–0.05 *M* Tris (pH 7.4) and filtered through 6 layers of cheesecloth and 1 layer of Miracloth. The homogenate was centrifuged at 2000*g* for 5 min. The cellular pellet was washed three times with Earle's balanced salt solution and pelleted by centrifugation at 2000*g*. Nuclei and chromatin were then prepared as previously described for HeLa S_3 cells. The protein/DNA ratios of these rat liver chromatin preparations are 2.0.

† From the Departments of Biochemistry (G.S.S., R.J.M., J.L.S., and J.D.) and Chemistry (E.J.G. and P.D.A.), University of Florida, Gainesville, Florida 32610. *Received December 20, 1974.* These studies were supported by Research Grants GB38349 from the National Science Foundation, GM20535 from the National Institutes of Health, F73UF from the American Cancer Society, and ORO-3982-31 from the Atomic Energy Commission.

Isolation of DNA. DNA was prepared from nuclei of exponentially growing HeLa S_3 cells and from rat liver nuclei by the method of Marmur (1961). The DNA was further purified by treatment with ribonuclease A for 30 min at 37° (50 μg/ml) and Pronase for 2 hr at 37° (50 μg/ml) and was then extracted twice with phenol. Enzymes were purchased from Sigma Inc., St. Louis, Mo.

Dissociation, Fractionation, and Reconstitution of Chromatin. All procedures were carried out at 4°. Chromatin was dissociated in 3 *M* NaCl–5 *M* urea–0.01 *M* Tris (pH 8.3), and DNA was pelleted by centrifugation at 150,000*g* for 48 hr. The supernatant containing the chromosomal proteins was fractionated into histone and non-histone chromosomal protein fractions by the QAE-Sephadex chromatographic method of Gilmour and Paul (1970) as previously described by Stein et al. (1972). Chromatin was then reconstituted as described by Bonner and coworkers (Bekhor et al., 1969; Huang and Bonner, 1965). DNA, non-histone chromosomal proteins, and histones were combined in 3 *M* NaCl–5 *M* urea–0.01 *M* Tris (pH 8.3) at a DNA/histone/non-histone chromosomal protein ratio of 1:2:2 (w/w). Chromatin was reconstituted by gradient dialysis against 5 *M* urea–0.01 *M* Tris (pH 8.3) containing successively decreasing amounts of sodium chloride. After complete removal of sodium chloride the reconstituted chromatin was pelleted by centrifugation at 20,000*g* for 30 min, resuspended in 0.01 *M* Tris (pH 8.3), and again pelleted by centrifugation at 20,000*g* for 30 min. Details of the procedure have been extensively characterized by Kleiman and Huang (1972).

Extraction of Histones. Chromatin samples containing 500 μg of DNA as chromatin were suspended in 2 ml of 0.25 *N* HCl by homogenization with a Dounce type homogenizer (A pestle), extracted for 20 hr at 4° with agitation by shaking throughout the extraction procedure, and pelleted by centrifugation at 10,000*g* for 15 min. Chromatin was then reextracted for an additional 30 min with 1 ml of 0.25 *N* HCl and pelleted by centrifugation at 10,000*g* for 30 min. The combined dilute mineral acid extracts were precipitated by addition of 9 volumes of acetone. After 12 hr at 4° the precipitates were pelleted by centrifugation at 15,000*g* for 15 min, washed successively with 5 ml of acetone and 5 ml of ether, and then dried in a vacuum desiccator. The acetone and ether washes were each followed by centrifugation at 15,000*g* for 15 min.

Polyacrylamide Gel Electrophoretic Fractionation of Chromosomal Proteins. A. HISTONES. Histones were fractionated electrophoretically according to charge and molecular weight on 0.6 × 9 cm, 15% polyacrylamide gels containing 2.5 *M* urea, according to the method of Panyim and Chalkley (1969). Fifty micrograms of histone (1 mg/ml in 0.9 *N* acetic acid–15% sucrose) were applied to each gel. The amount of protein was determined by the method of Lowry (1951). Electrophoresis was carried out for 4 hr at 2 mA/gel using 0.9 *N* acetic acid as the upper and lower tray buffer. Histone gels were stained overnight in 0.1% Amido Black–20% ethanol–7% acetic acid and destained for 15 min in 7% acetic acid using a Canalco "quick gel destainer".

B. TOTAL CHROMOSOMAL PROTEINS. Chromatin samples (500 μg of DNA as chromatin) were solubilized in 1 ml of 1% SDS[1]–1% β-mercaptoethanol–0.01 *M* sodium phosphate (pH 7.0) and dialyzed for 12 hr against 1000 volumes of 0.1% SDS–0.1% β-mercaptoethanol–0.01 *M* sodium phosphate (pH 7.0) at 22°. Sucrose was added to a final concentration of 15%, and the samples were heated in boiling water for 5 min. Fifty-microliter aliquots containing 50 μg of chromosomal proteins were electrophoresed on 0.6 × 7.5 cm, 7.5% polyacrylamide gels containing 0.1% SDS for 7 hr at 8 mA/gel in a running buffer of 0.1% SDS–0.1 *M* sodium phosphate–5 m*M* EDTA (pH 7.0). The gels were fixed overnight in 12% trichloroacetic acid–40% ethanol–7% acetic acid, stained for 5 hr at 37° in 0.25% Coomassie Brilliant Blue in 40% ethanol–7% acetic acid, and electrophoretically destained using 10% ethanol–7% acetic acid as a buffer.

Both histone and non-histone chromosomal protein gels were scanned at 600 nm in a Beckman Acta 2 spectrophotometer equipped with a linear gel transport. The areas under the peaks in the gel scan were integrated with a planimeter to determine the relative amounts of protein in each band or in different regions of the gel. Within the narrow range of protein concentrations used in these electrophoretic fractionations, the amounts of Amido Black and Coomassie Brilliant Blue stains bound (A_{600}) varied in a linear fashion with the amount of protein applied to the gels as determined by the method of Lowry (1951).

Selective Dissociation of Histones from Chromatin. Histones were selectively dissociated from chromatin by the method of Smart and Bonner (1971), using the ionic detergent sodium deoxycholate. Chromatin suspended in 0.0025 *M* Tris (pH 8.0) was diluted with the same buffer so that a final volume of 10 ml and a final concentration of 10 A_{260} units would be obtained after the addition of sodium deoxycholate. The required amount of 0.025 *M* sodium deoxycholate–0.0025 *M* Tris (pH 8.0) was added dropwise while stirring vigorously on a Vortex mixer. Each 10-ml chromatin sample was gently layered on 2 ml of 1.2 *M* sucrose–0.0025 *M* Tris (pH 8.0) and centrifuged in a 50 Ti Spinco rotor for 16 hr at 50,000 rpm. The pellet containing the partially dehistonized chromatin was then analyzed for DNA and chromosomal protein content. In agreement with Smart and Bonner (1971), the order of histone extraction with sodium deoxycholate was: slightly lysine rich, arginine rich, and then very lysine rich histones (Stein et al., 1974b). This order was observed in native as well as in reconstituted chromatin.

Preparation of Escherichia coli RNA Polymerase. RNA polymerase was prepared from mid-log phase *E. coli* (Grain Processing Corporation, Muscatine, Iowa) utilizing the procedure of Berg et al. (1971). The enzyme was purified to the fraction 5 stage which includes chromatography on DEAE-cellulose.

Transcription of Chromatin and DNA in Vitro. Chromatin and DNA were transcribed under conditions in which the template is rate limiting and reinitiation is prohibited (Cedar and Felsenfeld, 1973; Hyman and Davidson, 1970); 40 μg of chromatin or 40 μg of DNA was incubated at 37° for 15 min in 0.5 ml containing 10 m*M* Tris (pH 7.9), 1 m*M* $MnCl_2$, 0.08 m*M* each of ATP and GTP, 0.02 m*M* UTP and 25 μCi of [^{3}H]UTP (14 Ci/mmol, Schwarz/Mann, Inc.), and 2.4 units of *E. coli* RNA polymerase. Under these conditions transcription is initiated but elongation is blocked due to the absence of CTP. Ammonium sulfate (0.16 ml of a 1.69 *M* solution) was then added to a final concentration of 0.4 *M* to prevent further initiation, and elongation was started by the addition of CTP and

[1] Abbreviations used are: SDS, sodium dodecyl sulfate; Mes, 2-(*N*-morpholino)ethanesulfonic acid.

$MgCl_2$ to final concentrations of 0.063 and 5 m*M*, respectively. Incorporation of radioactivity into acid precipitable material was determined at various times during the elongation phase of the assay, as follows: 100-μl aliquots of the reaction mixture were withdrawn and precipitated with 5 ml of 10% trichloroacetic acid for 15 min. The precipitates were collected on 0.45-μ nitrocellulose (Millipore) filter discs and washed with 45 ml of 10% trichloroacetic acid. The filters were then solubilized in 1 ml of Cellosolve (ethylene glycol monoethyl ether) in a 20-ml liquid scintillation counting vial; 15 ml of scintillation cocktail containing Liquifluor, toluene, and Triton X-100 (0.126:2:1) was added to each vial prior to counting.

Determination of Circular Dichroism Spectra of Chromatin. Circular dichroism spectra of native and reconstituted HeLa S_3 chromatin in 0.25 m*M* EDTA (pH 7.0) at a concentration of 1 A_{260} were measured in a Jasco J-20 spectrometer. All measurements were carried at 4° using a Lauda K-2/R constant temperature water circulator and a dry nitrogen purge. The optical density ratio 400/258 nm did not exceed 0.03 indicating that light scattering effects did not significantly alter the circular dichroism spectra.

Determination of the Binding of a Reporter Molecule to Chromatin. Equilibrium dialysis studies were carried out in 10 m*M* 2-(*N*-morpholino)ethanesulfonic acid buffer (Mes) (pH 6.2) at 4°. These studies employed Visking dialysis tubing (26/100 Nojax casings) cut into 20-cm strips and carefully cleaned by boiling in 50% aqueous ethanol–5 m*M* EDTA–50 m*M* sodium bicarbonate for 1 hr. This procedure was repeated three times. The membranes were then boiled in deionized water for 5 hr. This procedure was repeated six times. The clean membranes were stored at 4° in deionized water containing 2% chloroform. The dialysis experiments were conducted in a pair of plexiglass blocks with ten shallow cylindrical depressions cut into each (3 × 0.25 cm). A clean membrane was placed between the cells to form two chambers. Each half of the plexiglass block, prior to placement of the membrane, was lightly coated with silicon grease to prevent leakage. The blocks were securely fastened and the top of the cells was sealed by placing a strip of lightly greased Parafilm over the top of the plexiglass block. Equilibration was allowed to take place for at least 20 hr at 4° during which time the dialysis blocks were gently shaken. (Time studies showed that the reporter molecule was equilibrated in less than 10 hr). The solution on the side of the cells containing only the small molecule was pipetted into a cuvet and the absorbance at 335 nm (i.e., at λ_{max} of the reporter) was recorded using a Gilford 240 spectrophotometer.

Preparation and Assay of ATP:polynucleotidylexotransferases. ATP:polynucleotidylexotransferase was purified from 4-day-old TMS waxy, WF9X Bear 38 maize seedlings (Bear Hybrid Corn Co., Decatur, IL) by the procedure of Mans and Huff (manuscript submitted) through gradient elution from DEAE-cellulose. The ATP:polynucleotidylexotransferase was purified from a post-ribosomal supernatant fraction from exponentially growing HeLa S_3 cells by the method of Mans and Stein (1974) through stepwise elution from DEAE-cellulose. A standard reaction mixture (0.1 ml) contained 70 m*M* Tris-HCl (pH 8.8), 10 m*M* dithiothreitol, 1.2 m*M* [8-^{14}C]ATP (1.5 Ci/mol), 1 m*M* $MnCl_2$, 100 μg/ml of bovine serum albumin, either 33 μg/ml of maize enzyme or 66 μg/ml of HeLa enzyme protein, and the indicated amount of primer. The reaction was initiated by addition of enzyme and incubation at 30°. At times indicated aliquots were withdrawn and the AMP incorporated into acid-insoluble material was determined on filter paper disks as described previously (Walter and Mans, 1970). *Escherichia coli* tRNA, [8-^{14}C]ATP, and unlabeled nucleotides were purchased from Schwarz/Mann, Inc. Protein concentration was determined spectrophotometrically by the method of Warburg and Christian (1941).

Table I: Chromosomal Protein and DNA Contents of Native and Reconstituted Chromatin.[a]

Sample	Protein/DNA (w/w)
Native HeLa	1.94
Reconstituted HeLa	1.92
Native rat liver	1.79
Reconstituted rat liver	1.85

[a] Histones were extracted with 0.25 *N* HCl as described under Materials and Methods. Nucleic acids were hydrolyzed with 1 *N* perchloric acid at 90° for 30 min and the non-histone chromosomal proteins were solubilized in 1 *N* NaOH. The amounts of protein in the histone and non-histone chromosomal protein fractions were assayed by the method of Lowry et al. (1951) and the amount of DNA in the nucleic acid fraction was determined by Burton's modification of the diphenylamine reaction (Burton, 1956). Each value represents an average of at least 5 determinations and the range of values did not exceed 5%.

Results

Composition of Native and Reconstituted HeLa S_3 Cell and Rat Liver Chromatin. Initially, the protein/DNA ratios of native and reconstituted HeLa S_3 cell and rat liver chromatin preparations were compared. Table I indicates that when HeLa S_3 cell and rat liver chromatins are dissociated, fractionated, and reconstituted as described under Materials and Methods the protein and DNA contents of the reconstituted chromatin preparation are identical with those of the native chromatin from which the DNA, histones, and non-histone chromosomal proteins are derived.

To determine whether there are significant variations in the quantities of specific molecular weight classes of chromosomal proteins in native and reconstituted HeLa S_3 cell and rat liver chromatin preparations, chromosomal proteins were fractionated electrophoretically according to molecular weight on SDS polyacrylamide gels. The polyacrylamide gel electrophoretic banding patterns of the chromosomal proteins from native and reconstituted HeLa S_3 cell chromatins are shown in Figure 1. Peaks S, U, V, and W represent histone polypeptides while the remaining peaks represent non-histone chromosomal proteins. Peak S contains F_1 histones which migrate anomalously in the 37,000 molecular weight region of the gel while peak U contains F_3 and F_{2b} histones, peak V contains F_{2a2} histones, and peak W contains F_{2a1} histones. A comparison of Figure 1A and B clearly establishes that within the limits of resolution afforded by SDS polyacrylamide gel electrophoretic fractionation the relative amounts of protein in the principal molecular weight classes of chromosomal proteins, histones as well as non-histone chromosomal proteins associated with the genome of native and reconstituted HeLa S_3 cell chromatin are quantitatively and qualitatively indistinguishable. This contention is further supported by the data in Table II. It should be noted, however, that there may be microheterogeneity of individual molecular weight classes of chromosomal proteins and differences at this level are not accounted for by the present data. To further define the histones as-

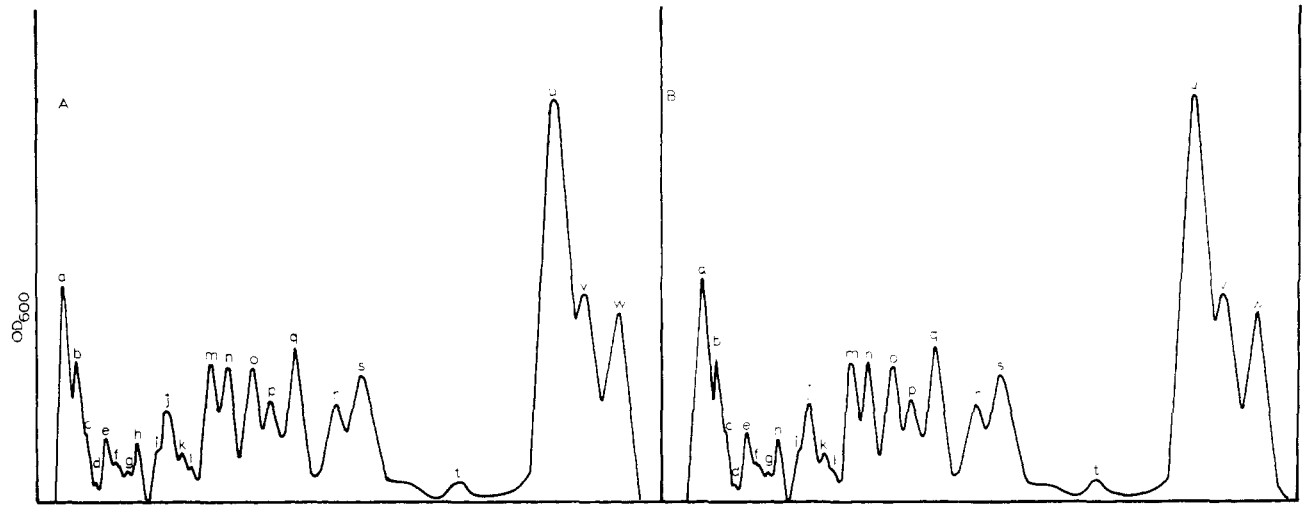


FIGURE 1: SDS polyacrylamide gel electrophoretic profiles of total chromosomal proteins from (A) native and (B) reconstituted HeLa S_3 cell chromatin.

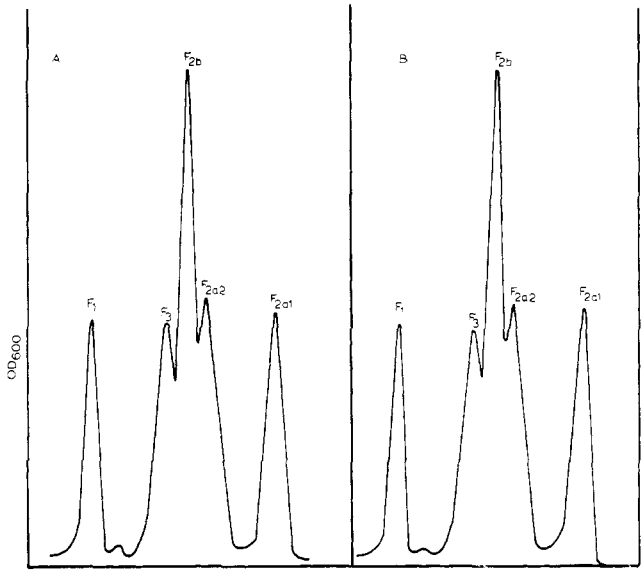


FIGURE 2: Histones extracted from native (A) and reconstituted (B) HeLa S_3 cell chromatin fractionated electrophoretically on 15% polyacrylamide gels containing 2.5 *M* urea and 0.9 *N* acetic acid (pH 2.5).

Table II: Relative Amounts of Histones and Non-Histone Chromosomal Proteins in Native and Reconstituted HeLa S_3 Cell Chromatin.

Sample	Non-Histone Proteins/Histone	Percentage of Total Histones				
		F_1	F_3	F_{2b}	F_{2a_2}	F_{2a_1}
Native chromatin	1.19	13.3	17.2	33.2	17.5	19.4
Reconstituted chromatin	1.22	12.4	18.7	33.7	19.3	19.1

[a] The non-histone chromosomal protein/histone ratios in native and reconstituted HeLa S_3 cell chromatin were obtained by integrating the areas under the absorbance scans shown in Figure 1. The relative amounts of protein in the various histone fractions from native and reconstituted HeLa S_3 cell chromatin were obtained by integrating the areas under the absorbance scans shown in Figure 2.

sociated with the genome of native and reconstituted HeLa S_3 cell chromatin, histone polypeptides were extracted with dilute mineral acid as described under Materials and Methods and electrophoresed on acetic acid–urea polyacrylamide gels by the high-resolution method of Panyim and Chalkley (1969). Figure 2 and Table II demonstrate the quantitative and qualitative similarity of the five principal histone classes from native and reconstituted HeLa S_3 cell chromatin fractionated in this manner.

Figure 3 shows the SDS polyacrylamide gel electrophoretic banding patterns of chromosomal proteins from native (3A) and reconstituted (3B) rat liver chromatin. Peaks P (F_1), S (F_3 and F_{2b}), T (F_{2a2}), and U (F_{2a1}) represent histone polypeptides while the remaining peaks represent nonhistone chromosomal proteins. Consistent with the similarity of proteins associated with the genome of native and reconstituted HeLa S_3 cell chromatin, the chromosomal polypeptides of native and reconstituted rat liver chromatin are qualitatively and quantitatively indistinguishable.

Binding of Histones in Native and Reconstituted HeLa S_3 Cell Chromatin. One method to define the manner in which histones are associated with other genome components in native and reconstituted HeLa S_3 cell chromatin is to assess their extractability with the ionic detergent sodium deoxycholate. We have previously shown that the probe provides a valid indication of histone binding in HeLa S_3 cell chromatin since increasing concentrations of sodium deoxycholate between 0.005 and 0.1 *M* selectively extract progressively increased amounts of histone polypeptides without releasing DNA or non-histone chromosomal proteins (Smart and Bonner, 1971; Stein et al., 1974b). In Figure 4 the effect of sodium deoxycholate concentration on the release of histones from native and reconstituted chromatin preparations is compared. The similar extractability of histones from native and reconstituted chromatin with concentrations of sodium deoxycholate between 0.001 and 0.1 *M* suggests that the binding of these basic chromosomal polypeptides is not significantly altered during dissociation, fractionation, and reconstitution of chromatin. It should be indicated that the sensitivity of this assay is sufficient to detect variations in histone binding during defined periods of the cell cycle and variations in several rat tissues as a function of age (Stein et al., 1973). Differences in histone binding of less than 10% are reproducibly resolvable by this procedure.

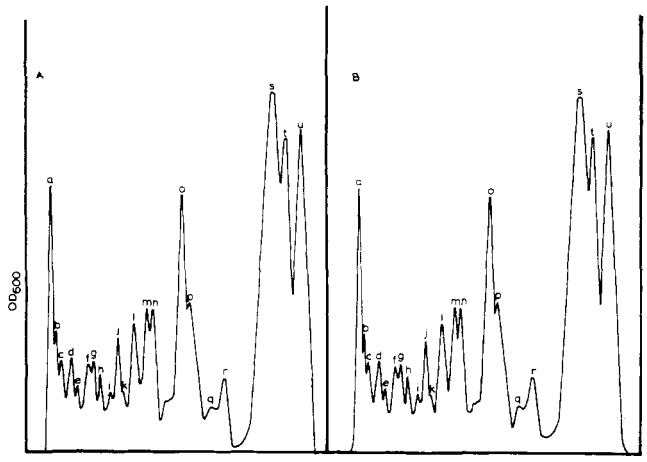


FIGURE 3: Polyacrylamide gel electrophoretic profiles of total chromosomal proteins from (A) native and (B) reconstituted rat liver chromatin.

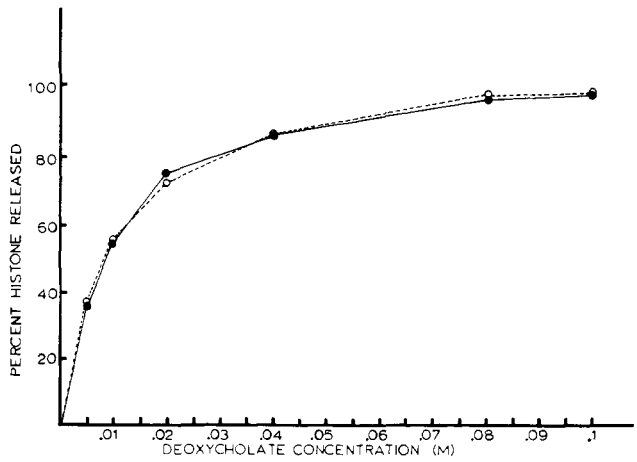
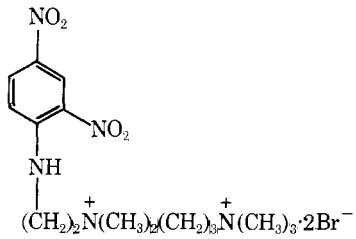


FIGURE 4: Dissociation of histones from native (O) and reconstituted (●) HeLa S_3 cell chromatin. Each point represents a minimum of four determinations and the range of values did not exceed 4%.

Transcription of Native and Reconstituted HeLa S_3 Cell Chromatin. Native and reconstituted chromatin and DNA were transcribed under conditions in which the template is rate limiting and reinitiation is prohibited, as described by Cedar and Felsenfeld (1973). Unsheared chromatin preparations were used in these assays since in our hands shearing enhanced transcription in a nonspecific manner. Figure 5 shows the absence of significant variations in the transcriptional properties of native and reconstituted HeLa S_3 cell chromatin. These findings suggest that in both native and reconstituted chromatin preparations a similar number of sites on the DNA is available for initiation and the time course of elongation is similar.

Binding of Reporter Molecules in Native and Reconstituted HeLa S_3 Cell Chromatin. Structural properties of the DNA double helix in chromatin are modified in a defined manner by the association of DNA with chromosomal proteins. Such protein–DNA interactions should be reflected by variations in the binding of "reporter molecules". The interaction of nitroaniline-labeled diammonium salts (reporter molecules) with nucleic acids has been extensively studied by Gabbay and coworkers (Gabbay, 1969; Passero et al., 1970; Gabbay and Sanford, 1974; Adawadkar et al, 1975). Evidence has been presented which is consistent with the intercalation of the dinitroaniline ring of **1** between base



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pairs of DNA with the diammonium side chain lying in the minor groove of the helix (Gabbay and Sanford, 1974; Adawadkar et al., 1975). The binding of this reporter molecule to native and reconstituted HeLa S_3 cell chromatin was compared, and the maximum number of reporter molecules bound per base pair was found to be similar in native and reconstituted chromatin ($\bar{n}_{max}$ equals 0.071 and 0.072, respectively). Approximately one reporter molecule is bound per seven base pairs which indicates that in each case approximately 7% of the phosphate groups are exposed and

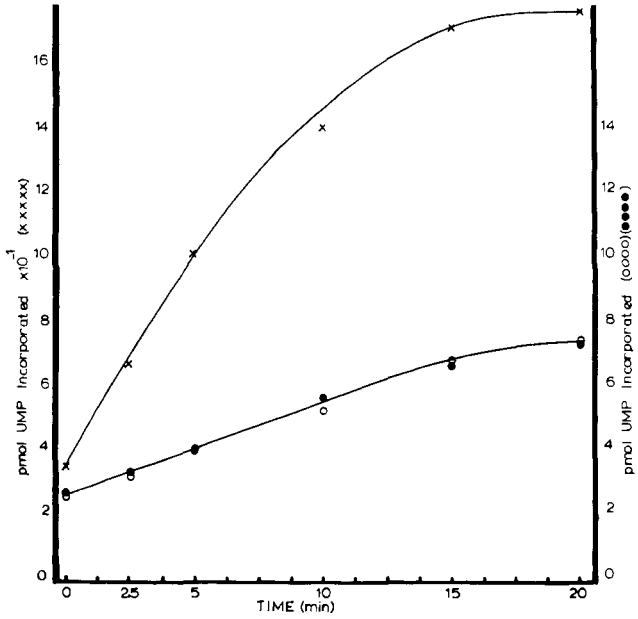

FIGURE 5: Transcription of native (O) and reconstituted (●) chromatin and DNA (×) under conditions where reinitiation by *E. coli* RNA polymerase is prohibited.

capable of interacting with this reporter molecule. While the primary mechanism by which this probe interacts with the DNA in chromatin has not been unequivocally established, the present results clearly demonstrate that its binding to DNA in native and reconstituted chromatin are essentially equivalent.

Circular Dichroism Spectra of Native and Reconstituted HeLa S_3 Cell Chromatin. A second probe used to assess the structural integrity of chromatin following dissociation, fractionation, and reconstitution was circular dichroism (CD). The CD spectra of native and reconstituted HeLa S_3 cell chromatin in the spectral range from 215 to 300 nm is shown in Figure 6. The maximum ellipticity for both native and reconstituted chromatin is 4500 at 272 nm. The point of crossover for both chromatin preparations is at 258 nm. Furthermore, the spectra of native and reconstituted chromatin are identical in the region of the negative ellipticity band of DNA at 245 nm as well as in the peptide chromophore region at approximately 220 nm.

Free DNA Ends in Native and Reconstituted HeLa S_3

Table III: Primer Activity of Native and Reconstituted Chromatin for Poly(A) Synthesis by Maize ATP: Polynucleotidylexotransferase.

Line No.	Primer	Specific Activity[a] 30 min (nmol/mg)	60 min (nmol/mg)	90 min (nmol/mg)
1	None	6.2[b]	17.1[b]	18.9[b]
2	*E. coli* tRNA (4.1)[c]	398	1013	1614
3	Native chromatin (1.1)	2.9	<0.1	3.4
4	Native chromatin (5.5)	10.3	15.4	37.7
5	Reconstituted chromatin (1.1)	1.9	4.4	12.3
6	Reconstituted chromatin (5.5)	8.4	23.3	35.6
7	Nat. chromatin (1.1) + tRNA (4.1)	407	988	1566
8	Reconst. chromatin (1.1) + tRNA (4.1)	430	992	1612
9	Nat. chromatin (2.7) + reconst. chromatin (2.7)	12.0	16.7	46.0
10	Calcd native (2.7) + reconst. chromatin (2.7)	9.4	19.4	36.7

[a] Specific activity is defined as the primer dependent nmoles of AMP incorporated into acid-insoluble material per mg of enzyme protein in a standard reaction mixture incubated at 30° for the times indicated. [b] Specific activity was calculated from the AMP incorporated without added primer. [c] Numbers in parentheses indicate primer concentration as A_{260}/ml.

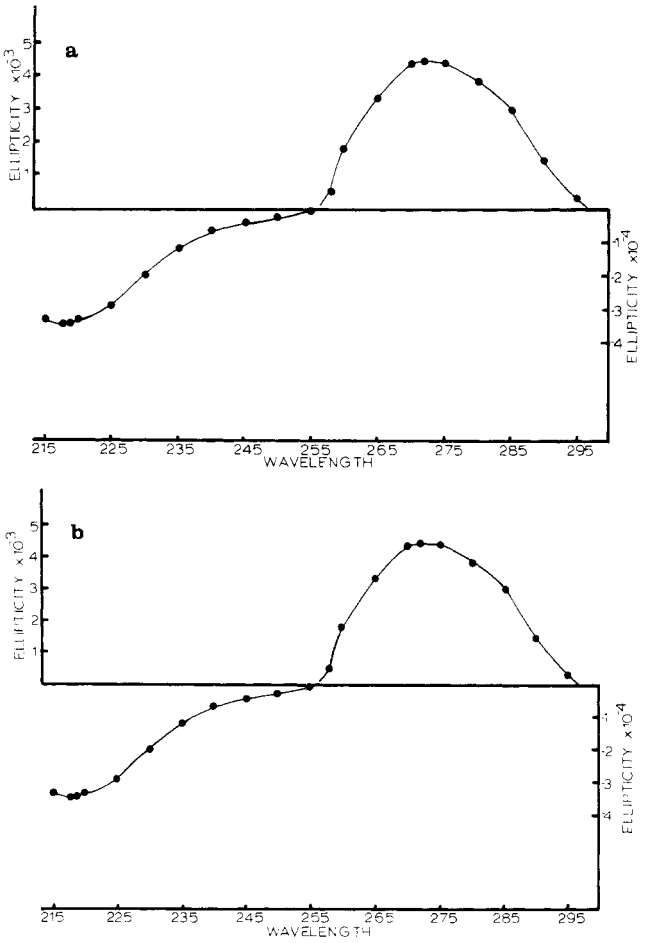

FIGURE 6: Circular dichroism spectra of (A) native and (B) reconstituted HeLa S_3 cell chromatin.

Cell Chromatin. Native and reconstituted chromatins were compared as primers for poly(A) synthesis by the ATP: polynucleotidylexotransferase purified from maize seedlings (Mans and Huff, manuscript submitted). This enzyme sequentially adds AMPs to the 3′-hydroxyl terminus of either single-stranded RNA (Mans and Walter, 1971) or deoxyoligomer primers (Mans, 1971; Mans and Huff, manuscript submitted) and, therefore, should detect any single-stranded deoxyoligomers generated in the reconstitution of HeLa chromatin. Data in Table III show that native and reconstituted chromatin do not differ significantly in their ability to prime poly(A) synthesis. The apparent differences between native and reconstituted chromatin shown in lines 3 and 5 are insignificant. This is supported by the similar levels of priming activity observed at higher chromatin concentrations (compare lines 4 and 6). The relatively low priming activity of either chromatin preparation as compared with that of *E. coli* tRNA (Table III, compare lines 4 and 6 with 2) indicates that few DNA or RNA 3′-hydroxyl termini are available to fulfill the enzyme primer requirement. Neither chromatin preparation inhibited the tRNA-primed reaction (Table III, compare lines 7 and 8 with 2). The primer activity of the two preparations assayed together was almost that calculated from their activity assayed individually (Table III, compare lines 9 with 10). When assayed for poly(A) priming activity with the ATP:polynucleotidylexotransferase purified from HeLa cells (Mans and Stein, 1974), the native and reconstituted chromatin preparations were again similar (Table IV). The HeLa exotransferase differs significantly from the maize enzyme in two respects; it utilizes only RNA primers for poly(A) synthesis and it exhibits a significant endogenous primer activity (compare line 1 in Tables III and IV). Neither chromatin preparation exhibited a significant poly(A) priming activity (Table IV, lines 3 and 4) suggesting that neither preparation contained exposed RNA.

Table IV: Primer Activity of Native and Reconstituted Chromatin for Poly(A) Synthesis by HeLa ATP:Polynucleotidylexotransferase.

Line No.	Primer	Specific Activity[a] 30 min (nmol/mg)	60 min (nmol/mg)	90 min (nmol/mg)
1	None	8.4[b]	64[b]	168[b]
2	*E. coli* tRNA (4.1)[c]	143	612	1236
3	Native chromatin (2.2)	1.3	6.6	1.0
4	Reconstituted chromatin (2.2)	3.3	2.3	−5.2

[a] Specific activity is defined as primer dependent nmoles of ATP incorporated into acid-insoluble material per mg of enzyme protein in a standard reaction mixture at 30° for the time indicated. [b] Specific activity was calculated from the AMP incorporated without added primer. [c] Numbers in parentheses indicate primer concentration as A_{260}/ml.

Discussion

Several lines of evidence are presented which suggest that chromatin can be dissociated, fractionated, and reconsti-

tuted without significantly altering the composition or structural and transcriptional integrity of the nucleoprotein complex. The absence of quantitative or qualitative variations in the proteins–histones and non-histone chromosomal proteins of native and reconstituted chromatin is suggested by two types of high-resolution polyacrylamide gel electrophoretic analysis. These findings are consistent with previous studies which indicate that variations in the specific activities of defined molecular weight classes of chromosomal polypeptides do not occur during dissociation, fractionation, and reconstitution of chromatin (Stein and Farber, 1972). Several reports have appeared over the past few years purporting that components of the genome may be altered due to treatments incurred during the dissociation, fractionation, and reconstitution processes (Bekhor et al., 1974; Chae and Carter, 1974). However, in our hands we see no signs of protein degradation.

Utilizing a number of high-resolution criteria, we have found that the organization and functional properties of chromatin from the several tissues examined are preserved in reconstituted chromatin. Evidence presented for structural fidelity of reconstituted chromatin includes the binding of a reporter molecule with specificity for the minor groove of the DNA helix, the binding of histones, and the numbers of sites available for addition of nucleotides to DNA. Perhaps the strongest argument for structural fidelity of reconstituted chromatin is that the circular dichroism spectrum is identical with that of native chromatin. These findings are in agreement with other reports that the thermal denaturation properties (Bekhor et al., 1969) and susceptibility to thymine base damage by γ radiation are not altered in reconstituted chromatin (Roti Roti et al., 1974).

Previous studies have indicated fidelity of transcription in reconstituted chromatin. In these studies the kinetics of hybridization to DNA of RNA transcripts from native and reconstituted chromatin were compared (Gilmour and Paul, 1970; Paul and Gilmour, 1968; Bekhor et al., 1969; Spelsberg and Hnilica, 1970). However, the conditions under which hybridization was carried out limited the sensitivity of the assay to moderately or highly reiterated sequences. Other studies have shown that the template activities of native and reconstituted chromatin are quantitatively identical (Stein and Farber, 1972; Stein et al., 1972; Bekhor et al., 1969; Spelsberg and Hnilica, 1971). It should be noted, however, that in these studies differences in rates of initiation and elongation, as well as in numbers of initiation sites available, may exist but be masked. Findings presented in this paper show that when native and reconstituted chromatin are transcribed under conditions where reinitiation is prohibited the transcriptional activities of both chromatin preparations are identical.

Taken together the evidence presented in this communication strongly suggests, but does not by any means conclusively establish, fidelity of chromatin reconstitution. Establishing the validity of the reconstitution procedure is of key importance since it represents one of the most powerful tools for directly examining the roles of defined genome components in regulating the transcription of specific genetic sequences. Already this approach has been valuable in deciphering the regulation of globin (Paul et al., 1973; L. S. Hnilica, personal communication) and histone genes (Stein et al., 1975). Undoubtedly, it will continue to be of great importance in establishing the mechanisms by which the vast quantity of information contained within the eukaryotic genome is selectively transcribed.

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The Role of Superoxide Radical in the Autoxidation of Cytochrome *c*†

Robert H. Cassell‡ and Irwin Fridovich*

ABSTRACT: The net rate of autoxidation of ferrocytochrome *c* was decreased by ferricytochrome *c*. Superoxide dismutase accelerated this autoxidation to a limit and overcame the inhibitory effect of ferricytochrome *c*. This was the case whether the autoxidation was observed in the presence or in the absence of denaturants, such as alcohols or urea, and whether the superoxide dismutase used was the Cu^{2+}-Zn^{2+} enzyme from bovine erythrocytes or the Mn^{3+}-enzyme from *Escherichia coli*. It can be deduced that the autoxidation of ferrocytochrome *c*, under a variety of conditions, generates O_2^- which can then dismute to $H_2O_2 + O_2$ or can reduce ferricytochrome *c* back to ferrocytochrome *c*. Superoxide dismutase, by accelerating the dismutation of O_2^-, prevents the back reaction and thus exposes the true rate of reaction of ferrocytochrome *c* with molecular oxygen.

Alcohols have been shown to facilitate the autoxidation of ferrocytochromes *c* and c_1 (Kaminsky et al., 1971; Yu et al., 1974). Higher alcohols were more effective in this regard than were lower homologs, and unbranched alcohols were more effective than the corresponding branched isomers. All of this is not surprising in view of the structure of ferrocytochrome *c* (Takano et al., 1973), in which the heme is enclosed in a hydrophobic crevice, and on the assumption that the situation in cytochrome c_1 is not very different. Thus alcohols could open the heme crevice both by partitioning into it and by changing the solvent properties of the bathing medium. The more hydrophobic the alcohol, the more effectively should it thus expose the heme and speed its reaction with dissolved oxygen. But what is the reduction product of oxygen in this autoxidation? If there is no aggregation of the ferrocytochrome prior to oxidation, and if the only oxidative change is the univalent conversion of ferroheme to the corresponding ferriheme, then the oxygen must be reduced to the superoxide radical (O_2^-).[1] If this were the case, it would create an interesting situation because O_2^- is a potent reductant of ferricytochrome *c* (McCord and Fridovich, 1968, 1969; Ballou et al., 1969; Land and Swallow, 1971). The autoxidation of ferrocytochrome *c* might therefore become self-limiting, since as ferricytochrome *c* accumulated, it would intercept an increasing fraction of the O_2^- generated. Ferricytochrome *c* would therefore have the apparent effect of inhibiting the autoxidation of ferrocytochrome *c*. In contrast, superoxide dismutase would, by catalytically scavenging O_2^- (McCord and Fridovich, 1969), prevent the reduction of the ferricytochrome *c*, and would thus expose the true rate of autoxidation. The following report demonstrates these effects and thus establishes that the autoxidation of ferrocytochrome *c* generates O_2^-.

Experimental Section

Materials. Horse heart cytochrome *c*, type III and type VI, was obtained from the Sigma Chemical Company, St. Louis, Mo. Bovine erythrocyte superoxide dismutase (SOD) was purchased from Truett Laboratories, Dallas, Texas, and was freed of carbonic anhydrase by passage through the affinity column described by Whitney (1974). Manganisuperoxide dismutase from *Escherichia coli B*, prepared by the method of Keele et al. (1970), was kindly provided by Dr. F. J. Yost, Jr. SOD was assayed by the method of McCord and Fridovich (1969). Reagent grade 1-butanol was purchased from Mallinckrodt Chemical Works, St. Louis, Mo., and 1-propanol from J. T. Baker Chemical Company, Phillipsburg, N.J. Deionized water was used throughout.

Methods. Cytochrome *c* concentrations were determined spectrophotometrically as described by Massey (1959). The concentration of oxygen was initially close to 0.2 m*M*. Since this exceeds the concentration of ferrocytochrome *c* by a factor of 5 there could not have been significant depletion of oxygen during the reactions observed.

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‡ Medical Scientist Trainee of the National Institutes of Health, Bethesda, Md.

[1] Abbreviations used are: O_2^-, superoxide anion; SOD, superoxide dismutase.