Attempted Separation of Transcriptively Active and Inactive Chromatin by Hydroxylapatite Thermal Chromatography†

Jeffrey E. Saffitz and Arnold I. Caplan*

ABSTRACT: Chromatin and purified DNA were fractionated by hydroxylapatite thermal chromatography. Fractions of varying thermal stability were tested for the proportions of transcribed sequences and repetitive sequences relative to the unfractionated genome. The first 80–85% of either total chromatin or purified DNA eluted from hydroxylapatite contained the same proportion of hybridizable sequences as total DNA. The remaining 15–20% of chromatin eluting at the highest temperatures was depleted of transcribed sequences. Analysis of the 20% highest melting fraction of purified DNA showed that, while the first two-thirds of this fraction contained the same proportion of transcribed sequences as unfractionated DNA, the last third, comprising about 6% of total DNA, was depleted of active sequences. Although no major differences were detected in nonrepetitive sequence complexity of chromatin fractions, there was a correlation between relative thermal stability and repetitive sequence content in fractions of both chromatin and DNA separated by thermal chromatography. Fragments eluting at higher temperatures contained a greater proportion of repetitive sequences, as indicated by a rapidly renaturing component. Most likely, the latest eluting fractions from both chromatin and purified DNA were enriched for a nontranscribed, highly reiterated, G+C rich satellite component of the chicken genome.

In differentiated cells, a relatively small portion of the genome is transcribed into RNA (Brown & Church, 1971; Grouse et al., 1972). Insight into the regulation of differential gene expression could be provided were one able to study the structural and functional features which distinguish transcriptively active and inactive chromatin. Toward this end, several methods for fractionating chromatin have been developed (for a recent review, see Gottesfeld, 1977; for a list of chromatin fractionation references, see Saffitz & Caplan, 1978).

We have studied one such method developed by McConaughy & McCarthy (1972) in which chromatin is fractionated by hydroxylapatite chromatography on the basis of differential thermal stability. A relationship between chromatin thermal stability and template activity is suggested from comparisons of chromatin and DNA denaturation in low ionic strength solution (Huang & Bonner, 1962; Marushige & Ozaki, 1967; Paoletti & Huang, 1969; Li & Bonner, 1971) and from observations that condensed chromatin denatures at higher mean temperatures than extended chromatin (Frenster, 1965; Duerksen & McCarthy, 1971). McConaughy & McCarthy (1972) provided evidence that, in fact, chick erythrocyte chromatin fragments eluting from hydroxylapatite at lower temperatures were enriched for template active sequences, while more thermally stabilized chromatin was devoid of transcribed genes.

Our initial studies examined the hydroxylapatite thermal chromatography of chromatin and DNA and the effects of elution buffer and fragment size on elution profiles. A fractionation system was developed in which chromatin and DNA demonstrated thermal denaturation features similar to those seen in low ionic strength solution and in which protein mediated thermal stabilization of chromatin DNA was maximized (Saffitz & Caplan, 1978). In this report, we studied the distributions of transcribed vs. nontranscribed sequences, and repetitive vs. nonrepetitive sequences within fractions of chromatin and DNA separated by hydroxylapatite thermal chromatography. In addition, the sequence complexity of each fraction relative to the total genome was examined to determine whether a true sequence fractionation occurs during hydroxylapatite thermal chromatography.

This technology has been applied to a complex and interesting developing muscle culture system from embryonic chick which provides the potential to study temporal phenomena regarding chromatin structure and function as they relate to the establishment and maintenance of a well defined genetic program. Our data indicate that the proportion of transcribed sequences in fractions comprising 80–85% of either total chromatin or purified DNA was equivalent to the unfractionated genome. The remaining 15–20% fraction, eluting at the highest temperature, was relatively depleted of transcribed sequences. A correlation between thermal elution temperature and the proportion of repetitive sequences was demonstrated with both chromatin and DNA; fragments eluting at higher temperatures were enriched for rapidly reassociating sequences. However, sequence complexity analysis of chromatin fractions suggests that no significant fractionation of nonrepetitive sequences occurs during hydroxylapatite thermal chromatography.

Materials and Methods

Methods for the preparation of developing muscle cultures, the labeling of chromatin DNA in culture, and the preparation of chromatin from in vitro and in vivo muscle tissue are provided in the preceding communication (Saffitz & Caplan, 1978). Muscle culture chromatin and DNA were fractionated on hydroxylapatite after shearing chromatin at either 15 000 or 30 000 psi[1] in a French pressure cell (Aminco). In all experiments, 0.025 M CsPB was used as elution buffer. In ex-

† From the Department of Biology and Developmental Biology Center, Case Western Reserve University, Cleveland, Ohio 44106. *Received January 4, 1978.* Supported by grants from the Muscular Dystrophy Association of America, the March of Dimes (National Foundation), and the National Institutes of Health. J.E.S. was generously supported by funds from a training grant (HD-00020).

[1] Abbreviations used: psi, pounds per square inch; CsPB, cesium phosphate buffer; $NaDodSO_4$, sodium dodecyl sulfate; NaPB, sodium phosphate buffer; T_m, mean temperature of thermal elution.

0006-2960/78/0417-3487$01.00/0

periments comparing chromatin with purified DNA, DNA was prepared from an aliquot of the same chromatin used in the comparable experiment. Procedures for shearing chromatin, production of CsPB, preparation of DNA from chromatin and the hydroxylapatite fractionation of chromatin and DNA are all detailed in the preceding communication.

After hydroxylapatite chromatography, fragments eluting within certain temperature ranges were pooled to form thermal fractions of either chromatin or purified DNA. The proportion of transcribed sequences within each thermal fraction was determined by RNA in excess hybridization using total cell RNA from day 15 embryonic chick muscle. The proportion of repetitive and nonrepetitive sequences within each thermal fraction was assessed by DNA reassociation kinetics with total DNA used as kinetic driver. Chromatin thermal fraction sequence complexity was determined by examining the self-driven DNA reassociation kinetics. The larger amounts of labeled chromatin required for this experiment were obtained from day 15 embryonic chick muscle tissue labeled in ovo. These procedures are described below.

Labeling of Chromatin in Ovo. Windows were cut in the shells of day 3 chick embryos and sealed with Scotch tape. On days 6 through 13, each embryo received 0.05 mL of sterile Tyrode's solution containing 10 μCi of [^{3}H]thymidine delivered by syringe through the taped window and placed directly on the embryo. At day 15, leg and thigh muscles were harvested, chromatin was prepared and sheared, and hydroxylapatite thermal fractionation was carried out as described for muscle culture chromatin, except that a column containing 20 mL of hydroxylapatite was employed. The resulting thermal fractions were processed for self-driven reassociation experiments. Cultured muscle chromatin was employed in all other experiments.

Processing of Chromatin and DNA Thermal Fractions. DNA in pooled hydroxylapatite thermal fractions (from either chromatin or purified DNA) was prepared for hybridization or reassociation experiments in the following manner. Fractions were dialyzed against 0.1 M NaCl, 0.05 M Tris (pH 7.4), 0.005 M EDTA (NTE buffer) at 4 °C to remove elution buffer. Macromolecules were precipitated with 2.5 volumes of ethanol at −20 °C for 24 h after 1 mg of purified *E. coli* RNA carrier had been added to each fraction. Precipitates were collected at 4 °C by centrifugation, resuspended in NTE buffer, and incubated at 37 °C with 10 μg/mL RNase B (boiled for 10 min to destroy DNase activity) and then with 25 μg/mL Pronase or Proteinase K (EM Laboratories) for an additional 2 h at 37 °C. $NaDodSO_4$ was added to 1% and the samples were extracted twice with an equal volume of freshly distilled phenol (saturated with NTE buffer) and once with chloroform:octanol (8:1, v/v) at room temperature. The extracted DNA was sheared at 30 000 psi in a French pressure cell (to standardize DNA fragment size) and dialyzed against distilled water for 24 h at 4 °C. After evaporation to dryness, samples were dissolved in NTE buffer and passed over Sephadex G-100 equilibrated with water at room temperature. Excluded fragments were collected, evaporated to dryness, and dissolved in either 0.4 M NaPB for hybridization reactions or 0.12 M NaPB for reassociation reactions at a concentration of approximately 4000–5000 cpm/μL.

Preparation of RNA. Total cell RNA for use in hybridization experiments was purified from day 15 embryonic chick leg and thigh muscle by a modified method of Aviv & Leder (1972). Fresh muscle was dissected in Tyrode's solution containing 100 μg/mL sodium heparin. Tissue was mechanically homogenized in 0.1 M NaCl, 0.1 M Tris (pH 9.0), 0.003 M EDTA (homogenization buffer) containing 1% $NaDodSO_4$ and 100 μg/mL sodium heparin. The homogenate was extracted several times with freshly distilled phenol (saturated with homogenization buffer at pH 9.0) and a final time with chloroform:isoamyl alcohol (100:1, v/v). Macromolecules were precipitated with 2 volumes of ethanol at −20 °C overnight. The precipitate was collected by centrifugation, resuspended in 0.1 M NaCl, 0.05 M Tris (pH 9.0), 0.001 M $MgCl_2$, and incubated with 10 μg/mL DNase I (Worthington Laboratories) for 1 h at 37 °C. $NaDodSO_4$ was added to 1% and EDTA to 0.005 M and the sample was extracted once with phenol and once with chloroform:isoamyl alcohol (100:1, v/v) and ethanol precipitated. The precipitate was resuspended in 20 mL of 0.02 M sodium acetate buffer (pH 5.1) containing 19.56 g of optical grade CsCl (ρ = 1.74 g/mL) and centrifuged at 35 000 rpm for 72 h at 24 °C in a Type 60 Ti rotor and Model L-5 ultracentrifuge (Beckman Instruments). This procedure resulted in quantitative pelleting of highly purified RNA (Glisin et al., 1974). Sucrose gradient analysis before and after pelleting through dense CsCl gradients failed to detect any degradation of RNA during this procedure.

The purified RNA was resuspended in 0.1 M sodium acetate buffer (pH 5.1) and passed over a column of Sephadex G-25 equilibrated with the same buffer at room temperature. Excluded material was passed over a column of Chelex-100 in 0.1 M sodium acetate buffer (pH 5.1) and ethanol precipitated. RNA was stored as an ethanol precipitate at −20 °C. The precipitate was resuspended in 0.4 M NaPB in high concentrations for use in hybridization experiments.

Preparation of DNA. Unlabeled, total DNA for use as kinetic driver in reassociation experiments was isolated from day 15 embryonic chick brain. Nuclei were prepared by homogenizing tissue at 4 °C in NTE buffer containing 1% Nonidet P-40 (Shell Chemicals). Purified nuclei were resuspended in NTE buffer containing 1% $NaDodSO_4$ at room temperature and extracted several times with freshly distilled phenol saturated with NTE buffer, pH 7.4, and once with chloroform:isoamyl alcohol (100:1, v/v). After ethanol precipitation, the sample was incubated with 25 μg/mL RNase B for 2 h at 37 °C and then with 25 μg/mL Proteinase K for an additional 2 h. The sample was brought to 1% with $NaDodSO_4$, extracted twice with phenol and once with chloroform:isoamyl alcohol, and ethanol precipitated. The purified DNA was sheared at 30 000 psi in a French pressure cell to yield fragments of approximately 300 base pairs (Ordahl et al., 1976; Saffitz & Caplan, 1978). Sheared DNA was excluded from Sephadex G-100 equilibrated with NTE buffer at room temperature and then passed over Chelex-100 in NTE buffer. Purified sheared DNA was stored as an ethanol precipitate at −20 °C. The precipitate was resuspended in 0.12 M NaPB for use in reassociation experiments.

Preparation and Analysis of DNA–RNA Hybridization Reactions. Processed thermal fraction DNA from hydroxylapatite chromatography of chromatin and DNA was hybridized with high concentrations of total muscle cell RNA according to the method of Ordahl & Caplan (1976). An aliquot of thermal fraction DNA in 0.4 M NaPB was flame sealed in a capillary and heated to 100 °C for 10 min to denature duplex structures. The denatured DNA was mixed with purified RNA in 0.4 M NaPB at approximately 18 mg/mL; aliquots of 20 μL were flame sealed in capillaries, immediately frozen in a dry ice–ethanol bath, and stored at −20 °C. Reactions were begun by placing capillaries in a 65 °C water bath in which they were incubated until the desired $C_{R0}t$ had been reached. Reaction $C_{R0}t$ (moles of RNA nucleotides × seconds per liter) values were determined according to Britten & Kohne (1968) and converted to 0.12 M NaPB equivalent $C_{R0}t$

TABLE I.[a]

starting material	thermal fraction	proportion of total cpm (%)	temp range of elution (°C)	mean temp of elution (°C)
15 000 psi sheared chromatin	A	15	55–80	77.5
	B	30	80–85	82.5
	C	34	85–95	89.0
	D	21	95–urea	
DNA from 15 000 psi sheared chromatin	A	10	55–75	72.5
	B	30	75–80	78.0
	C	39	80–85	82.0
	D	21	85–urea	89.0
30 000 psi sheared chromatin	A	17	55–80	76.5
	B	36	80–85	82.5
	C	31	85–95	88.0
	D	16	95–urea	
DNA from 30 000 psi sheared chromatin	A	10	55–75	72.0
	B	33	75–80	78.0
	C	38	80–85	82.5
	D	19	85–urea	88.0

[a] A summary of the features of thermal fractions obtained from hydroxylapatite thermal chromatography of 15 000 psi and 30 000 psi sheared chromatin and DNA.

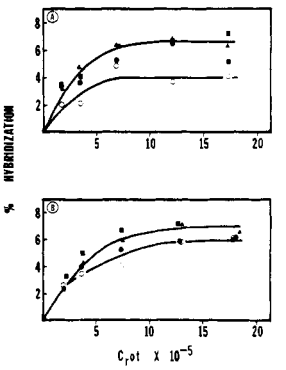

FIGURE 1: Hybridization of DNA from thermal fractions A through D from hydroxylapatite thermal chromatography of 15 000 psi sheared chromatin (A) or DNA (B) to total cell RNA from day 15 embryonic chick leg and thigh muscle tissue. Reactions, run in 0.4 M NaPB at 65 °C, contained approximately 8000 cpm of thermal fraction DNA and 300 μg of purified RNA in a volume of 20 μL; RNA:DNA ratio = 7500:1. At various times, reactions were stopped and treated with S_1 nuclease. An aliquot was analyzed on hydroxylapatite to determine the percent of the total cpm represented as total duplex (DNA-DNA duplex + RNA-DNA hybrids). Another aliquot was RNase treated and analyzed to measure the percent of total cpm present as DNA-DNA duplex. Each value represents the net hybridization level obtained by subtracting the DNA-DNA duplex value from the total duplex value. Reaction rates have been normalized to 0.12 M NaPB "equivalent $C_{R0}t$"; data are expressed as the percent of the total cpm representing hybrid structures vs. equivalent $C_{R0}t$ developed. A (●); B (■); C (▲); D (○).

by an empirical formula established by Britten (1969).

Hybridization reactions were analyzed by treating diluted samples with S_1 nuclease to digest unreacted nuclei acids and single stranded tails according to the method of Ordahl & Caplan (1976). Following treatment with S_1 nuclease, each sample was divided into equal aliquots to which were added 0.6 mL of 0.12 M NaPB. One aliquot was brought to 0.25% with $NaDodSO_4$, incubated at 75 °C for 5 min, and strand fractionated on hydroxylapatite in 0.12 M NaPB at 65 °C (Kohne & Britten, 1971). Hydroxylapatite binding material in this aliquot represented both DNA-RNA hybrids and DNA-DNA duplexes. The remaining S_1 nuclease treated aliquot served as a control in which only DNA-DNA duplex structures were quantitated according to the following procedure. Following incubation at 75 °C for 5 min, RNase B was added to 50 μg/mL and the sample was incubated for 1 h at 65 °C. Under these conditions of high RNase concentration and temperature, RNase destroys RNA-DNA hybrids by digesting the RNA component (Shultz et al., 1973). The sample was brought to 0.25% with $NaDodSO_4$ and strand fractionated on hydroxylapatite to assay for DNA-DNA duplex structures.

Hydroxylapatite strand separation was accomplished by applying samples to columns containing 0.5 mL settled bed volume of hydroxylapatite (Hypatite-C, Clarkson Chemicals) at 65 °C. Nonbinding material was eluted with 0.12 M NaPB and bound material (DNA-DNA duplex and/or DNA-RNA hybrid) was eluted with 0.5 M NaPB. The eluates were quantitated directly in a liquid scintillant containing Triton X-100 according to Ordahl & Caplan (1976). A net hybridization value was obtained for each reaction by subtracting the DNA-DNA duplex value (S_1 nuclease + RNase treated aliquot) from the total duplex value (S_1 nuclease only treated aliquot). Data are expressed by plotting the net hybridization value for each reaction vs. the total equivalent $C_{R0}t$ developed by that reaction.

Preparation and Analysis of DNA Reassociation Kinetics Reactions. DNA prepared from hydroxylapatite thermal fractions was reassociated with unlabeled total DNA and the kinetics of reassociation were monitored by standard hydroxylapatite methods (Britten & Kohne, 1968; Kohne & Britten, 1971). Trace amounts of labeled thermal fraction DNA were mixed with unlabeled total DNA in 0.12 M NaPB and sealed in capillaries. Reactions were begun by heating capillaries to 100 °C for 10 min. After incubation to the desired C_0t (moles of DNA nucleotides × seconds per liter, determined according to Britten, 1969), the reactions were diluted into 1.0 mL of 0.12 M NaPB at 65 °C, applied to a hydroxylapatite column, and strand separated. The data are expressed by plotting the percent double strands present within each reaction, determined by hydroxylapatite binding, vs. the C_0t developed by each reaction.

Self-driven reassociation reactions were set up by simply diluting thermal fraction DNA to the desired concentration with 0.12 M NaPB and flame sealing aliquots in capillaries. Reactions were begun by heating to 100 °C for 10 min, and, after the desired C_0t was reached at 65 °C, hydroxylapatite analysis was performed.

Results

Chromatin and DNA Hydroxylapatite Thermal Fractions. Labeled, cultured muscle chromatin was sheared at either 15 000 or 30 000 psi. Sheared chromatin and corresponding DNA samples were fractionated by hydroxylapatite chromatography. The resulting elution profiles are shown in Figures 3A and 3B of the preceding communication. Comparative features of the thermal fractions are summarized in Table I.

Transcribed Sequence Distribution in Chromatin and DNA Thermal Fractions. Total cell RNA saturation hybridization kinetics of 15 000 psi sheared chromatin thermal fractions are shown in Figure 1A. Thermal fractions A through C, representing approximately 80–85% of total chromatin cpm, attained roughly equivalent saturation values of 6.5 to 7.0%. Thermal fraction D, exhibiting a saturation value of about 4%, was relatively depleted of hybridizable sequences. Figure 1B illustrates a similar experiment with hydroxylapatite thermal fractions of purified DNA from 15 000 psi sheared chromatin.

Carbon-13 Nuclear Magnetic Resonance as a Probe of Side Chain Orientation and Mobility in Carboxymethylated Human Carbonic Anhydrase B†

Antonius J. M. Schoot Uiterkamp, Ian M. Armitage,* James H. Prestegard, John Slomski, and Joseph E. Coleman*

ABSTRACT: ^{13}C NMR spectra of [1-^{13}C]- and [2-^{13}C]carboxymethyl His-200 human carbonic anhydrase B have been obtained as a function of pH and in the presence and absence of the active site Zn(II) or Cd(II) ion. Chemical shifts of the 1-^{13}C show that the carboxyl is sensitive to two ionization processes, with apparent pK_as of 7.2 and 9.9, respectively. These are assigned to the deprotonation of the N^{π} of His-200 and the breaking of the coordination bond between the carboxyl oxygen and the Zn(II) ion, respectively. Assignment of the lower pK_a to that of the N^{π} is supported by the observation of this same ionization in the chemical shift of the 2-^{13}C resonance showing the signal from the methylene carbon to undergo the same upfield shift as is observed on the ionization of the N^{π} in N^{τ}-carboxymethylated histidine. The high pH ionization process is not reflected in the resonance of the methylene carbon. No changes in the chemical shifts vs. pH are observed for both the [1-^{13}C]- and [2-^{13}C]carboxymethyl apocarbonic anhydrase, suggesting that the pK_a of the N^{π} has shifted at least 1 pH unit to acid pH, and must reflect significant conformational changes in the active center. Cd(II) carboxymethyl carbonic anhydrase shows ^{13}C chemical shifts identical with those of the apoenzyme. Since the Cd(II) at the active site is known to bond external donor groups very weakly, the data suggest that the changes in conformation are related to Zn(II)–carboxylate coordination. Changes in the mobility of the carboxymethyl group have been assessed by a relaxation analysis which relates the relative line widths of the central and outer lines of the [^{13}C]methylene triplet to the internal rotational motion of the group relative to the protein. At neutral pH this group shows no internal motion, compatible with its coordination to the active site metal ion. At pH 10.6 significant internal motion is present, compatible with breaking of the coordination bond in competition with ^{-}OH binding.

Human erythrocytes contain two forms of carbonic anhydrase (carbonate hydrolyase, EC 4.2.1.1), a low and a high activity isozyme, designated carbonic anhydrase B and carbonic anhydrase C, respectively (Lindskog et al., 1971). These zinc metalloenzymes have a mol wt of ~30 000 and crystal structures at a resolution of 2.2 Å for B and 2.0 Å for C are known (Liljas et al., 1972; Kannan et al., 1975). Carbonic anhydrase catalyzes the reversible hydration of CO_2 as well as a number of other hydrolysis and hydration reactions involving carbonyl groups, including the hydrolysis of several esters (Pocker & Watamori, 1971).

All isozyme and species variants studied thus far show a pH–rate profile sigmoid to high pH with midpoints from pH 6.9 to 7.5 depending on isozyme and conditions, e.g., the presence of anions (Lindskog, 1966; Coleman, 1967a). To a first approximation the pH–rate profile is represented by a single ionization. The ionization resulting in the active form of the enzyme is thought to be that of either a Zn(II)-coordinated water molecule or an amino acid side chain adjacent to the metal ion (see Lindskog & Coleman, 1973, for discussion).

The B isozyme is much more sensitive to inhibition by anions than the C isozyme (Maren et al., 1976). The structure of the B isozyme also differs from that of the C isozyme in that the active site cleft of the B isozyme contains a histidine residue at position 200 which can be selectively carboxymethylated at the N^{τ} position by iodo- or bromoacetate. Carboxymethylation of His-200 results in a shift of the pK_a of the activity-linked group to pH ~9.0. Maximum activity of the carboxymethylated enzyme is ~20% that of the native enzyme. The carboxymethylation of the enzyme requires that iodo- or bromoacetate first be bound in reversible fashion to the active center, a reaction that depends on the presence of the metal ion (Whitney et al., 1967; Bradbury, 1969). These observations were combined in a specific model for the carboxymethylation reaction by Coleman (1971) in which the carboxyl of the reagent coordinates the metal ion, which leaves the N^{τ} of His-200 in a position for nucleophilic attack at the iodinated carbon. After reaction the carboxymethyl group remains coordinated to the Zn(II) ion at neutral pH, while ^{-}OH competes successfully at high pH regenerating the active enzyme and accounting for the shift in the apparent pK_a of the activity-linked group to pH ~9. Powerful anionic inhibitors like CN^{-} and sulfonamide compete with the carboxyl for the coordination site at neutral pH for which there is spectral evidence using the d–d transitions of the Co(II) enzyme (Coleman, 1971, 1975). In order to further test the validity of this hypothesis we have carried out a ^{13}C NMR study of carboxymethylated human carbonic anhydrase B (Cm HCAB[1]), using iodoacetate enriched in either the carboxyl carbon (Cm[1-^{13}C]HCAB) or the methylene carbon (Cm[2-^{13}C]HCAB).

The metal ion dependence of the chemical shifts of these carbons as functions of pH support an interaction of the car-

† From the Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06510, and the Department of Chemistry, Yale University, New Haven, Connecticut 06520. *Received January 13, 1978.* This work was supported by Grants AM 09070-14 and AM 18778-02 from the National Institutes of Health and PCM 76-82231 from the National Science Foundation. Acknowledgment is made to the Petroleum Research Fund administered by the American Chemical Society for partial support of this work. The high-field NMR work was supported by Grant No. 1-P07-PR 00798 from the National Institutes of Health, Division of Research Resources.

[1] Abbreviations used: HCAB, human carbonic anhydrase B; CmHCAB, carboxymethyl human carbonic anhydrase B.

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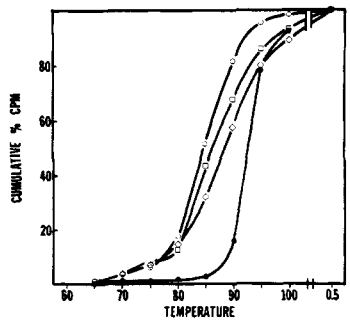

FIGURE 4: Hydroxylapatite T_m analysis of hybridization reactions of DNA of thermal fractions B and D from 15 000 psi sheared cultured muscle chromatin and unfractionated total DNA to total cell RNA from day 15 embryonic chick leg and thigh muscle tissue. Reactions were incubated to an equivalent $C_{R0}t$ of 75 000, treated with S_1 nuclease and applied to hydroxylapatite columns at 65 °C. After removing unbound single-stranded material, duplex structures were thermally eluted in 5 °C increments in 0.12 M NaPB. After elution in 0.12 M NaPB at 100 °C, remaining bound material was eluted with 0.5 M NaPB at 100 °C. Data are expressed as the cumulative percent of the total bound cpm eluting vs. temperature and are compared with the thermal elution profile of native chick DNA, sheared at 15 000 psi and thermally chromatographed under identical conditions. T_m's are 85.0, 86.5, 88.5, and 92.5 °C for duplex structures of thermal fraction B (○), unfractionated DNA (□), thermal fraction D (◇), and native chick DNA (●), respectively.

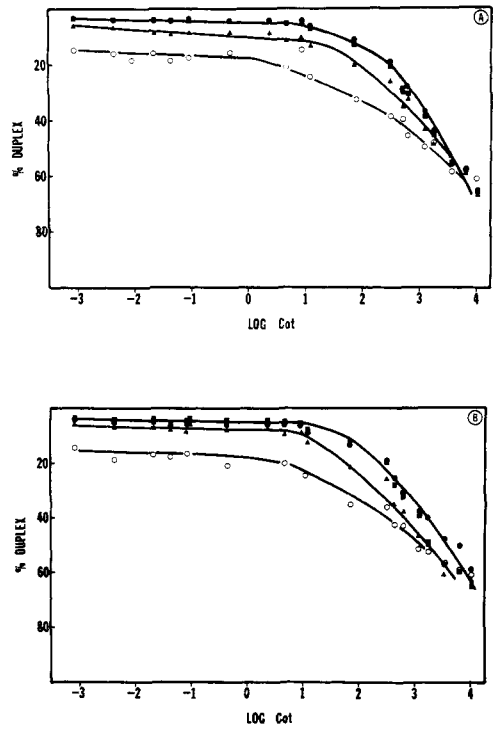

FIGURE 5: The reassociation of thermal fraction DNA from 15 000 psi sheared, cultured muscle chromatin (A) or DNA (B) kinetically driven by unlabeled total DNA. Reactions were carried out in 0.12 M NaPB at 65 °C in volumes ranging from 5 to 50 μL and concentrations of 0.08 μg/mL to 6.45 mg/mL. At various times, reactions were stopped and analyzed for duplex structures on hydroxylapatite. Data are presented as the percent of the total cpm represented as duplex structures, as determined by hydroxylapatite binding, vs. the log C_0t developed. A (●); B (■); C (▲); D (○).

(Martinson, 1973). Fraction D duplex material exhibited a T_m 4 °C lower than native DNA. This smaller difference was probably due to a higher inherent thermal stability and a greater proportion of DNA–DNA duplex within total duplex. Duplex formed by hybridizing total DNA with RNA exhibited a T_m 6 °C lower than native DNA. Predictably, this value was between those for thermal fractions B and D. Although these

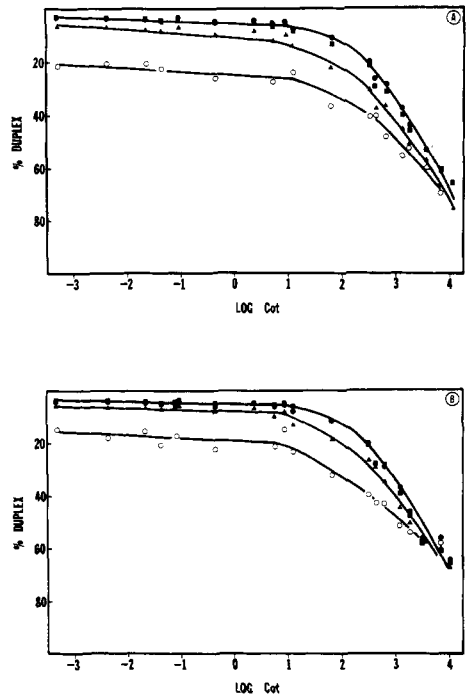

FIGURE 6: The reassociation of thermal fraction DNA from 30 000 psi sheared cultured muscle chromatin (A) or DNA (B) kinetically driven by unlabeled total DNA. Reaction conditions and analysis are identical with those of Figure 5. Data are expressed as the percent of the total cpm represented as duplex structures as determined by hydroxylapatite binding vs. the log C_0t developed. A (●); B (■); C (▲); D (○).

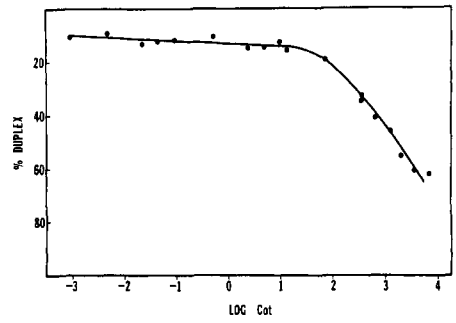

FIGURE 7: The reassociation of unfractionated [^{3}H]thymidine-labeled DNA kinetically driven by unlabeled total DNA. Reaction conditions and analysis were identical with those of Figure 5. Data are presented as the percent of the total cpm represented as duplex structures as determined by hydroxylapatite binding vs. the log C_0t developed.

T_m values were obtained from hybridization reactions in which the presence of repetitive DNA and high salt conditions would tend to promote base pairing mismatch (McCarthy & Church, 1970), they compare favorably with reported T_m differences in which only nonrepetitive DNA was hybridized (Brown & Church, 1971; Nieman, 1972; Liarakos et al., 1973).

Repetitive and Nonrepetitive Sequence Distribution in Chromatin and DNA Thermal Fractions. The total DNA driven reassociation kinetics of DNA from 15 000 psi sheared chromatin and DNA fractions and 30 000 psi sheared chromatin and DNA thermal fractions are illustrated in Figures 5A and 5B and Figures 6A and 6B, respectively, and may be compared with the reassociation kinetics of unfractionated DNA shown in Figure 7. These experiments investigated the possible correlation between chromatin and DNA hydroxylapatite thermal stability and sequence copy frequency.

DNA from specific thermal fractions exhibited similar reassociation profiles regardless of whether the fractions originated from 15 000 or 30 000 psi sheared chromatin or

TABLE III.[a]

starting material	thermal fraction	% repetitive sequences (C_0t = 10)
15 000 psi sheared chromatin	A	6
	B	7
	C	13
	D	23
DNA from 15 000 psi sheared chromatin	A	8
	B	7
	C	11
	D	23
30 000 psi sheared chromatin	A	6
	B	7
	C	13
	D	27
DNA from 30 000 psi sheared chromatin	A	8
	B	7
	C	12
	D	25
unfractionated total DNA		13

[a] A summary of the repetitive sequence DNA content of unfractionated DNA and DNA from thermal fractions operationally defined at C_0t = 10 in the presence of driving quantities of unlabeled total DNA. Thermal fractions were obtained from hydroxylapatite thermal chromatography of 15 000 psi and 30 000 psi sheared cultured muscle chromatin and DNA summarized in Table I. Reassociation kinetics are illustrated in Figures 5 through 7.

TABLE IV.[a]

starting material	thermal fraction	proportion of total cpm (%)	temp range of elution (°C)	mean temp of elution (°C)
15 000 psi sheared chromatin, 15 μg of chromatin DNA, 2.5 mL column bed volume	A	14	55–80	77.5
	B	30	80–85	83.0
	C	33	85–95	88.5
	D	23	95–urea	
15 000 psi sheared chromatin, 4.5 mg of chromatin DNA, 20 mL column bed volume	A	11	55–80	77.5
	B	30	80–85	82.5
	C	32	85–95	88.0
	D	27	95–urea	

[a] A summary of the features of thermal fractions obtained from the large scale hydroxylapatite thermal chromatography of 15 000 psi sheared chromatin from day 15 embryonic chick leg and thigh muscle tissue labeled in ovo. Data are compared with similar features from the standard thermal elution of 15 000 psi sheared cultured muscle chromatin.

DNA. Table III describes each fraction in terms of the proportion of repetitive sequences defined at a C_0t of 10. Fractions A and B contained 6 to 8% repetitive sequences, about half the proportion observed in total DNA. Fractions C contained 11 to 13% repetitive sequences, which approximates the composition of the whole genome. Fractions D reassociated to 23–27% at a C_0t of 10, indicating these fractions contained more than 1.5 times the amount of repetitive sequences in total DNA. Furthermore, fractions D contained a significant proportion of highly repeated sequences which reassociated at an extremely rapid rate.

The kinetics of reassociation illustrated in Figures 5 through 7 occurred a slower rate (half C_0t of about 1800) than some other published data (half C_0t of 500 to 1000, Rosen et al., 1973; DeJimenez et al., 1974). One factor responsible for the slower kinetics relates to differences in fragment size between the labeled thermal fraction DNA and the unlabeled driver DNA. A labeled probe smaller than the kinetic driver DNA results in a slower apparent rate of reassociation and a greater half C_0t of reaction (Britten & Kohne, 1968; Britten, 1969; Smith et al., 1975). Analysis of single-strand fragment size by alkaline sucrose gradient sedimentation revealed that various thermal fraction DNA fragments had a single-strand molecular weight approximately 0.75 times that of unlabeled driver (Studier, 1965). Applying this to the expression $K_{obsd} = K_d(L_t/L_d)$ (Smith et al., 1975) where K_{obsd} is the reciprocal half C_0t for the radioactive tracer (thermal fraction DNA), K_d is the reciprocal half C_0t of the driver DNA, and L_t and L_d are single-strand lengths of tracer and driver DNA, respectively, the half C_0t of the driver reaction becomes 1300. In addition, the driver DNA used in the present studies is probably smaller than fragments used in the other studies (Rosen et al., 1973; DeJimenez et al., 1974) which would further reduce the apparent discrepancy in rates of reaction.

Significant degradation of thermal fraction DNA during long term incubation of higher C_0t reactions would also result in an apparent reduction in reaction rate. However, such degradation did not occur as judged by Sephadex G-100 exclusion of labeled DNA. Sample reactions were begun by heating to 100 °C for 10 min and some were immediately passed over Sephadex G-100; an average of 97.9% of the labeled DNA was excluded. Other reactions were incubated to a C_0t of 12 000 (about 7 days) at 65 °C after which 95.8% was excluded. Thus, significant thermal degradation of reactants did not occur even after prolonged incubation at 65 °C.

Thermal Fraction Sequence Complexity. In the previous experiments, the kinetics of reassociation were determined exclusively by the driver (total) DNA regardless of thermal fraction sequence composition. In the following experiments the sequence complexity of 15 000 psi sheared chromatin thermal fractions was measured by following the self-driven kinetics of reassociation of each fraction. Under these circumstances, the rate of reassociation directly reflects the sequence complexity, that is, the number of different kinds of sequences.

This experiment required large quantities of labeled thermal fraction DNA to permit development of higher C_0t values. This was accomplished by isolating chromatin from day 15 embryonic chick leg and thigh muscle tissue labeled in ovo. Table IV compares features of the profile of approximately 4.5 mg (DNA content) of 15 000 psi sheared chromatin (specific activity = $1–1.2 \times 10^3$ cpm/μg) thermally eluted from a 20 mL bed volume hydroxylapatite column to one obtained by eluting a cultured muscle chromatin preparation (about 15 μg of DNA content) from a standard 2.5-mL hydroxylapatite column. The two melting systems produced essentially the same thermal fractions.

The self-driven kinetics of reassociation of 15 000 psi sheared chromatin thermal fractions are illustrated in Figure 8. Thermal fractions A, B, and C reassociated with kinetics similar to total DNA suggesting that the sequence complexity within each thermal fraction approximated that of the total genome. Fraction D appeared to reassociate at a lower C_0t than total DNA. However, this apparent difference was due to the high proportion of repetitive sequences in fraction D. Fraction D (Figure 8) is comprised of about 30% repetitive and 70% nonrepetitive sequences while total DNA (Figure 7) contains 13% repetitive and 87% nonrepetitive sequences, defined at a

C_0t of 10. Therefore, the concentration of nonrepetitive sequences in fraction D would be about 80% of the concentration of nonrepetitive sequences in an equal amount of total DNA. It follows then, that the C_0t developed by the nonrepetitive component of fraction D would be 80% of the C_0t developed by this component of total DNA when equal concentrations of the total samples are compared. If the reassociation of nonrepetitive sequences is defined as occurring beyond a C_0t of 10, then the half C_0t of reassociation of the nonrepetitive component of total DNA occurred at a C_0t of 3980 (from Figure 7). This value is comparable to the half C_0ts of reassociation of fractions A through C in Figure 8. The half C_0t of reassociation of fraction D nonrepetitive sequences occurred at a C_0t of 5010. Correcting this value for the lower relative concentration of nonrepetitive sequences in fraction D results in a value of about 4000 (5010 × 0.8). This value is close to that of total DNA indicating equivalency in nonrepetitive sequence complexity.

In summary, although thermal fractions varied regarding the relative proportion of repetitive and nonrepetitive sequences, the sequence complexity of the nonrepetitive component of each fraction is roughly equivalent to total DNA. No detectable sequence fractionation of nonrepetitive DNA occurred during hydroxylapatitie thermal fractionation of chromatin.

Discussion

Hybridization experiments performed on hydroxylapatite thermal fractions suggest that transcribed and nontranscribed chromatin do not possess sufficient differences in thermal stability to be separated by hydroxylapatite thermal chromatography. Chromatin thermal fractions A through C, representing 80–85% of total chromatin DNA, contained about the same proportion of hybridizable sequences as the unfractionated genome. Only chromatin fraction D appeared relatively depleted of transcribed sequences. That this relative depletion was more apparent in 30 000 psi sheared chromatin than 15 000 psi sheared material suggests an interspersion of transcribed and non-transcribed sequences.

Unlike chromatin fractions D, DNA fractions D hybridized to roughly the same levels as total DNA. The majority of these hybridizable sequences of DNA fractions D was localized to the lower melting component (fraction D_1). These results suggest that some nontranscribed sequences eluting in fractions A through C during purified DNA thermal fractionation are stabilized by chromatin protein such that they elute in fraction D_1 during chromatin thermal fractionation. However, the fact that fractions D_2 (latest eluting fragments) from *both* chromatin and DNA are depleted in transcribed sequences suggests that these nontranscribed sequences elute at the same relative thermal elution profile position due to inherent properties of the DNA and apparently independently of an association with chromosomal protein. These findings are consistent with data from total DNA driven reassociation studies. Regardless of the source (DNA or chromatin sheared at 15 000 or 30 000 psi), all thermal fractions D were enriched for rapidly reassociating sequences.

Perhaps the rapidly reassociating fragments of fractions D represent a nontranscribed, G+C rich satellite component of the chicken genome probably consisting of many copies of a short sequence arranged in tandem. Colbert et al. (1976) have identified a number of putative satellite components in the chicken genome comprising at most, 9–10% of total DNA. One component, comprising 3–5% of the genome, exhibited (a) a buoyant density of 1.712 g/mL on neutral CsCl compared with 1.698 for total DNA, (b) a G+C content of 53% compared with 39.6% for total DNA, and (c) a T_m 6–7 °C higher than for total DNA. Colbert et al. (1976) also identified a component comprising 3.9% of the genome which reassociated at an extremely rapid rate such that it bound to hydroxylapatite at "zero reaction time". Zero time binding probably occurs independently of molecular collisions in fragments containing many copies of the same short sequence in tandem and results from internal base pairing by a fold back mechanism (Britten, 1969). Thus Colbert et al. (1976) identified a zero time binding component comprising 3.9% of the chicken genome and a G+C rich satellite component also representing 3–5% of the genome. Thermal fractions D contain sequences which share these features. First, fractions D undoubtedly contain a high proportion of G+C rich fragments because of the high degree of hydroxylapatite thermal stability they exhibit. Second, fraction D averaged 19% of the total unfractionated DNA and 16.5% binding to hydroxylapatite at the lowest C_0t examined ($C_0t = 10^{-3}$) during reassociation studies. This extremely rapidly binding fraction would then represent 3.1% of the total genome (0.19 × 0.165) which is equivalent in amount to the zero binding time and G+C rich components identified by Colbert et al. Third, the depletion of transcribed sequences in fractions D_2 is consistent with the presence of a significant amount of very rapidly reassociating DNA. While RNA transcripts from the middle repetitive sequence component have been demonstrated in many systems, there is no evidence that the very highly repeated, satellite like DNA component is transcribed (Flamm et al., 1969; Davidson & Britten, 1973; McCall & Aronson, 1974; Greenberg & Perry, 1971).

It is therefore proposed that fractions D_2 from hydroxylapatite chromatography of chromatin and DNA are depleted in transcribed sequences as a result of an enrichment for a G+C rich, very rapidly reassociating, nontranscribed component of the chicken genome. That this component eluted from hydroxylapatite at the highest temperatures during both chromatin and purified DNA fractionation suggests that chromatin proteins do not play a significant role in conferring the high thermal stability characteristic of these fractions. On the other hand, chromatin fraction D_1 was depleted of transcribed sequences compared to DNA fraction D_1 presumably resulting from a shift of some nontranscribed sequences to a more thermally stabilized condition due to chromosomal protein.

Although a correlation between the proportion of repetitive sequences and thermal stability was demonstrated in hydroxylapatite thermal fractions, examination of the nonrepe-

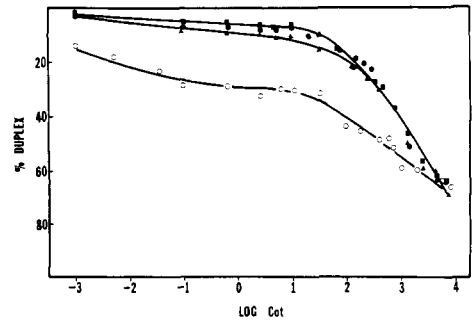

FIGURE 8: The self-driven kinetics of reassociation of DNA from thermal fractions obtained from the hydroxylapatite thermal chromatography of 15 000 psi sheared chromatin from day 15 embryonic chick leg and thigh muscle tissue labeled in ovo. Reactions were carried out in 0.12 M NaPB at 65 °C in volumes of 2 μL to 2 mL and DNA concentrations of 0.91 μg/mL to 4.4 mg/mL. At various times, reactions were stopped and analyzed on hydroxylapatite. Data are expressed as the percent of the total cpm represented as duplex structures as determined by hydroxylapatite binding vs. the log C_0t developed. A (●); B (■); C (▲); D (○).

titive component of chromatin fractions failed to demonstrate any major sequence fractionation. Within the limits of this technique, self-driven kinetics of reassociation suggested that 15 000 psi sheared chromatin thermal fractions contain approximately the same number of different kinds of sequences as unfractionated total DNA.

The hybridization data presented in this report are at variance with the data of McConaughy & McCarthy (1972). Several possibilities might account for this rather significant discrepancy. First, McConaughy & McCarthy selected the chicken erythrocyte as an experimental system, a system which represents a developmental end stage with a highly restricted and stable genetic program (Cameron & Prescott, 1963) in which only 1% of the genome hybridizes with erythrocyte RNA (McCarthy et al., 1973). Avian erythrocytes also contain the specialized histone H5 (Hnilica, 1972) which may play a role in maintaining the low template activity and highly condensed structure of erythrocyte chromatin (Billet & Handley, 1972). Embryonic muscle, on the other hand, represents a developing system with a much more complex genetic program which changes with time (Ordahl & Caplan, 1976). Thus, it might be predicted, a priori, that greater success would be obtained in fractionating erythrocyte chromatin. Second, McConaughy & McCarthy did not carry out thermal fractionation of purified DNA; if hybridization analysis of DNA fractionations revealed results similar to chromatin fractions, it would suggest the fortuitous elution of erythyrocyte template active chromatin sequences at low temperature. This would occur, for example, if the transcribed chromatin sequences happened to be of rather low G+C content. Third, the hybridization analysis of McConaughy & McCarthy is difficult to interpret. Little information is provided regarding control of DNA-DNA duplex formation and some values are probably overestimates. For example, a saturation value of more than 25% is reported for hybridization of total DNA with total RNA from adult chicken liver. Studies involving the mouse genome have shown no more than 5% of adult liver DNA is transcribed into RNA (Brown & Church, 1971; Grouse et al., 1972).

Our findings suggest that the only true and effective fractionation during hydroxylapatite thermal chromatography involves what is most likely a satellite component of the chick genome. In fact, this has been observed in numerous chromatin fractionation experiments in several systems, especially those having well defined and easily detected satellites. For example, Yasmineh & Yunis (1969) using the differential centrifugation technique of Frenster (1965) fractionated sheared chromatin from mouse brain and liver by differential sedimentation. The A+T rich satellite component was effectively separated from the other portion of the genome. In similar experiments with chromatin from the crab *Cancer majestis*, as well as mouse, Duerksen & McCarthy (1971) observed that both the poly[(A-T)] and G+C rich satellites and the mouse satellite were separable from the major portion of the genome. Thermal denaturation of the various chromatin fractions of the crab revealed a significant increase in T_m for satellite enriched components and a decrease in satellite depleted fractions compared with total chromatin. The same was true when DNA was isolated from total and fractionated chromatin and similarly studied. In a study of mouse hepatoma chromatin sheared by endogenous nuclease autodigestion and fractionated by the glycerol gradient technique of Murphy et al. (1973), Paul & Duerksen (1976) observed a fractionation of satellite sequences. McCarthy et al. (1973) demonstrated that extremely rapidly renaturing components of the *Drosophila* genome were separated after sheared chromatin was fractionated on steep sucrose gradients. Using the ECTHAM-cellulose ion exchange chromatography method of Reeck et al. (1972), Simpson (1975) observed that mouse satellite was separable from the remaining bulk of chromatin. And recently, using the hydroxylapatite fractionation procedure of McConaughy & McCarthy (1972), Pashev (1977) showed that, in mouse chromatin, satellite DNA preferentially eluted at high temperatures in spite of its A+T rich composition. These studies indicate that, in a variety of systems, chromatin fractionation techniques are effective in separating highly redundant satellite components which differ from the remaining chromatin in several important features. Probably, these satellites are not transcribed and therefore not associated with various indicators used to identify template active sequences (e.g., in vivo or in vitro synthesized RNA, endogenous or exogenous RNA polymerase binding, or hybridization with RNA). Perhaps much of the apparent success in fractionating chromatin over the past several years might not have resulted from separation of template active sequences from nontranscribed sequences but rather from separation of satellite components from nonsatellite components, as is most likely the case in the present studies.

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Nuclear Protein Modification and Chromatin Substructure. 1. Differential Poly(adenosine diphosphate) Ribosylation of Chromosomal Proteins in Nuclei versus Isolated Nucleosomes†

Chandrakant P. Giri,‡ Michael H. P. West, and Mark Smulson*

ABSTRACT: Poly(adenosine diphosphate-ribose) polymerase has been shown to modify a number of histone and nonhistone proteins. The species of proteins which were modified varied according to the conditions under which the reaction was carried out. Development of a two-dimensional gel system has allowed the identification of modified proteins not only in the nucleus as a whole, but also, for the first time, in nucleosomal fragments of chromatin generated by digestion with micrococcal nuclease. When ADP ribosylation was carried out in intact nuclei, H1 and H2B were found to be major acceptors, with H2A, H3, HMG, and M1-M4 proteins being modified to a lesser extent. After digestion with micrococcal nuclease, modified histones were associated preferentially with mononucleosomes and dimers, whereas higher oligomers displayed a wide variety of heavily modified nonhistone proteins. The specific activity of the enzyme was shown to increase with increasing nucleosome repeat number, and considerable activity was found in subnucleosomes. No H4 ADP ribosylation was detected in this study. The evidence relating to the modification of basic proteins was corroborated when the proteins isolated from mononucleosomes, dimers, and trimers were analyzed in a single-dimension electrophoresis, where quantitation was more easily achieved. This pattern of histone modification was shown to be dependent on chromatin being in its "native" conformation within the nucleus and emphasized the importance of interaction between the ADP-ribosylating system and the core particles. If the reaction was carried out with isolated nucleosomes, very little histone modification, with the exception of H1 and H3.1, occurred, but the ADP ribosylation of the HMG proteins, M1, and M4 was greatly enhanced.

The interactions between proteins and DNA play a vital role in determining the organization and expression of the genome. A knowledge of the ways in which these proteins can be modified by chromatin-associated enzymes is consequently of great importance in understanding the functioning of the nucleus.

Poly(ADP-Rib)[1] polymerase, a tightly bound chromatin enzyme, catalyzes the successive transfer of ADP-Rib units from NAD to nuclear proteins (see reviews by Hayaishi and Ueda, 1977; Smulson and Shall, 1976; Hilz and Stone, 1976). This results in the generation of short poly(ADP-Rib) chains, covalently attached to both histone and nonhistone chromosomal proteins. The enzyme may also cross-link proteins (Lorimer et al., 1977). The purified enzyme shows an absolute requirement for DNA, and addition of histones further stimulates the activity (Okayama et al., 1977; Yoshihara et al., 1977).

† From the Department of Biochemistry, Schools of Medicine and Dentistry, Georgetown University, Washington, D.C. 20007. *Received January 9, 1978; revised manuscript received April 17, 1978.* Supported by National Institutes of Health Grant CA 13195.

‡ Submitted to the Department of Biochemistry in partial fulfillment of the requirements for the Ph.D. degree. Present address: Department of Biology, University of Rochester, Rochester, N.Y. 14627.

[1] Abbreviations used are: ADP-Rib, adenosine diphosphate-ribose; Ado(P)-Rib-P and ψ-ADP-Rib, 2′-(5′-phosphoribosyl)-5′-AMP; NAD, nicotinamide adenine dinucleotide; $NaDodSO_4$, sodium dodecyl sulfate; Cl_3AcOH, trichloroacetic acid; $PhCH_2SO_2F$, phenyl methanesulfonyl fluoride; HMG, high-mobility group proteins; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; NHP, nonhistone protein; NMN, nicotinamide mononucleotide.

0006-2960/78/0417-3495$01.00/0