Hydroxylapatite Thermal Fractionation of Chromatin and DNA†

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ABSTRACT: Chromatin and DNA from developing muscle cultures were fractionated by hydroxylapatite thermal chromatography on the basis of differential thermal stability. A thermal chromatography system was developed in which protein mediated thermal stability of chromatin DNA was maximally expressed. The resulting chromatin and DNA elution profiles were similar to thermal denaturation profiles in low ionic strength solution. Additional studies showed this system was able to detect protein stabilization of DNA in in vitro nucleohistone preparations. Although some protein remained bound to hydroxylapatite during chromatin thermal elution, it did not affect the denaturation or elution behavior of free DNA on the same column. These studies show that fragments of chromatin or DNA can be segregated on the basis of differential thermal stability by hydroxylapatite chromatography.

It is generally believed that interactions between proteins and DNA in chromatin are responsible for the selective gene transcription seen in differentiated cells. Analysis of the molecular characteristics which distinguish template active from template inactive chromatin will likely shed light on the mechanisms controlling differential gene expression. Toward this end, attempts have been made to separate chromatin into transcribed and nontranscribed components. Methods of fractionation include differential centrifugation (Frenster et al., 1963; Frenster, 1965; Chalkley & Jensen, 1968; Yasmineh & Yunis, 1969; Yunis & Yasmineh, 1970; Duerksen & McCarthy, 1971; McCarthy et al., 1973; Murphy et al., 1973; Chesterton et al., 1974; Berkowitz & Doty, 1975; Burkholder & Weaver, 1975; Charles et al., 1975; Howk et al., 1975; Paul & Duerksen, 1976), differential solubility (Jensen & Chalkey, 1968; Marushige & Bonner, 1971; Billing & Bonner, 1972; Bonner et al., 1973; Gottesfeld et al., 1974, 1975, 1976; Arnold & Young, 1974; Pederson & Bhorjee, 1975), hydroxylapatite thermal chromatography (McConaughy & McCarthy, 1972), agarose gel exclusion chromatography (Janowski et al., 1972; McCarthy et al., 1973; Anderson et al., 1975; Pays & Flamand, 1976; Kreig & Wells, 1976), ECTHAM-cellulose ion-exchange chromatography (Reeck et al., 1972, 1974; Simpson & Reeck, 1973; Simpson & Polacow, 1973; Polacow & Simpson, 1973; Simpson, 1975; Howk et al., 1975; Levner et al., 1975; Keller et al., 1975) and others (Markov et al., 1975; Jalouzot et al., 1975).

Thermal fractionation of chromatin by hydroxylapatite chromatography, devised by McConaughy & McCarthy (1972), is based on observations that, in low ionic strength solution, chromatin denatures with a multitransitional curve at a higher mean temperature and over a wider temperature range than DNA (Marushige & Ozaki, 1967; Paoletti & Huang, 1969). These features of chromatin denaturation result from stabilization of the DNA double helix by chromosomal protein. Furthermore, in chromatin separated by shearing and differential centrifugation, highly condensed fractions exhibit higher mean temperatures of denaturation than extended fractions (Frenster, 1965; Duerksen & McCarthy, 1971). This implied relationship between thermal stability and template activity was investigated by McConaughy & McCarthy (1972) by eluting hydroxylapatite bound chromatin by a temperature gradient to yield fractions of varying thermal stability. Analysis of the fractions suggested that lower melting fragments were enriched for template active sequences while the more thermally stabilized fragments were devoid of transcribed genes.

We have studied hydroxylapatite thermal fractionation of chromatin and DNA and applied it to a developing muscle culture system from embryonic chick. The effects of different elution buffers on the T_m[1] and temperature ranges of elution of both chromatin and DNA were investigated. The importance of fragment size and its effect on features of chromatin and DNA elution profiles were also established. In addition, hydroxylapatite thermal elution of chromatin and DNA was compared with the thermal denaturation of chromatin and DNA in solution. The thermal elution of chromosomal proteins was also studied. A hydroxylapatite thermal fractionation system was developed in which the differences between chromatin and DNA thermal stability due to chromosomal protein were maximally expressed. A subsequent report will deal with the efficacy of this system in separating template active and template inactive chromatin.

Materials and Methods

Preparation of Developing Muscle Cultures. Cultures were established with myoblasts from the leg and thigh regions of day 12 White Leghorn chick embryos by the vortex method of Caplan (1976). Approximately 6.0–6.5 × 10^6 cells in a volume of 7.5 mL containing 7.5 μCi/mL [6-^{3}H]thymidine (9.0 Ci/mmol, New England Nuclear) were plated in each 100-mm gelatin coated Petri dish and incubated at 37.5 °C in a humidified atmosphere of 95% air–5% CO_2. Once myoblast fusion had progressed maximally (48–56 h after plating), the medium was replaced with fresh medium containing 70 μg/mL NaF, resulting in the nearly complete elimination of mononuclear cells (primarily fibroblasts). After an 18-h exposure to NaF and a brief recovery period, the myotubes morpho-

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

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logically and functionally resembled those of untreated cultures. Tissue was harvested after approximately 120 h of culture life, at which point the cultures represented a nearly homogeneous population of post fusion myoblasts undergoing rapid biochemical synthesis and accumulation of muscle specific gene products (Herrmann et al., 1970).

Preparation of Muscle Culture Chromatin. Chromatin was prepared from muscle culture material by a method modified from Marushige & Bonner (1966). All steps were performed at 4 °C. Cultures were washed with Tyrode's solution. Nuclei were prepared by homogenizing tissue in saline-EDTA (0.075 M NaCl, 0.024 M EDTA, pH 8.0) containing 1% Nonidet P-40 (Shell Chemicals) followed by centrifugation at 1000*g* for 15 min. After washing the pellets twice in saline-EDTA, the nuclei were suspended in 1.0 M sucrose in 0.01 M Tris (pH 8.0) and pelleted through 2.0 M sucrose in 0.01 M Tris (pH 8.0) at 25 000 rpm for 90 min at 4 °C in a Spinco SW27.1 rotor. The resulting gelatinous pellets were washed twice in 0.01 M Tris (pH 8.0). The final nuclear pellet was resuspended in the appropriate buffer for use in experiments and sheared.

Shearing was accomplished by passing the purified nuclear suspension through the valve of a French pressure cell under various pressures created by an Aminco Power Laboratory Press. Debris (containing less than 1% of the total cpm) was removed by centrifuging at 16 300*g* for 10 min. The supernatant containing sheared, purified chromatin in the appropriate buffer was poured off and used immediately.

Preparation of Chromatin from Embryonic Muscle Tissue. Chromatin used in optically monitored denaturation experiments was prepared from leg and thigh muscle tissue from day 15 chick embryos. Fresh tissue was dissected in Tyrode's solution, passed through a tissue press, and homogenized in saline-EDTA containing 1.5% Nonidet P-40. The homogenate was passed through four layers of cheesecloth and nuclei were pelleted by centrifugation at 1000*g* for 15 min. All subsequent steps were identical with those of the cultured tissue chromatin preparation.

Solubilized chromatin prepared by this procedure met purity standards established by others (Bonner et al., 1968). These included spectral ratios of $A_{260}/A_{280} = 1.65 \pm 0.05$, $A_{260}/A_{240} = 1.40 \pm 0.05$ and A_{320} less than 10% of the A_{260}. Purity was further assessed by demonstrating thermal denaturation characteristics consistent with published accounts, including denaturation profile shape, T_m, and percent hyperchromicity.

Preparation of DNA from Chromatin. In all cases in which hydroxylapatite elution profiles or optically monitored denaturation profiles of chromatin and DNA were directly compared, the DNA used was purified from an aliquot of chromatin used in the comparable experiment. The aliquot was adjusted to 0.1 M NaCl, 0.05 M Tris (pH 7.4), 0.005 M EDTA (NTE buffer) and incubated with 10 μg/mL RNase B (previously boiled for 10 min to destroy DNase activity) for 2 h at 37 °C. Boiled Pronase or Proteinase K (EM Laboratories) was added to 25 μg/mL and the sample was further incubated at 37 °C for 2 h. $NaDodSO_4$ was added to 1% and the sample was extracted twice with freshly distilled phenol (saturated with NTE buffer, pH 7.4) and once with chloroform: octanol (8:1, v/v). After dialysis against distilled water for 24 h at 4 °C, the DNA sample was adjusted to the appropriate buffer composition and studied either by hydroxylapatite thermal elution or optically monitored thermal denaturation.

Preparation of Elution Buffers. Sodium, potassium, and cesium phosphate buffers were used as elution buffers in hydroxylapatite thermal chromatography. NaPB and KPB were formed in the conventional manner by mixing equal volumes of equimolar monobasic and dibasic forms of either sodium or potassium phosphate. Such mixtures at 0.12 M had a pH of 6.7 at 20 °C.

CsPB was formed by reacting cesium carbonate and concentrated phosphoric acid according to the following equation:

$$3Cs_2CO_3 + 4H_3PO_4 \rightarrow 2Cs_2HPO_4 + 2CsH_2PO_4 + 3H_2CO_3$$

The reaction was driven to completion by gently heating the solution under a vacuum to remove CO_2 resulting from the equilibrium $H_2CO_3 \leftrightharpoons H^+ + HCO_3^- \leftrightharpoons H_2O + CO_2$. The solution was titrated to pH 6.7 at 20 °C with H_3PO_4 at which point it was assumed that equivalent proportions of the monobasic and dibasic forms of cesium phosphate existed in equilibrium.

Thermal Fractionation of Chromatin and DNA on Hydroxylapatite. The following procedure is based on methods established by Miyazawa & Thomas (1965) and modified by McConaughy & McCarthy (1972). Columns were fashioned from glass syringes and housed in a heat-resistant plastic vessel. Temperature was controlled by a Haake Model E-52 constant temperature circulator. Hydroxylapatite (Hypatite C, Clarkson Chemicals) was prepared by boiling crystals in 0.001 M NaPB and decanting off extremely fine particles. Column beds of 2.5 mL settled volume were routinely used. Chromatin or DNA, at 4 °C in the appropriate elution buffer, was applied to the column and equilibrated to 55 °C. After the initial passage, the effluent was again passed through the column and unbound material was removed by washing the bed with 5 column volumes of buffer. The temperature was increased by 5 °C to 60 °C and after equilibration, melted material was eluted with 2 column volumes of buffer. Two additional washes of 0.5 column volumes each were separately collected to determine whether complete elution of unbound material had occurred. Subsequently, the temperature was raised in 5 °C increments and elution carried out as described at each temperature level. After washing the column with elution buffer at 100 °C, remaining bound material was eluted first with 3 column volumes of 0.5 M NaPB (pH 6.5) and then with 3 column volumes of 8.0 M urea wash (8.0 M urea, 0.24 M NaPB, 0.01 M EDTA, pH 6.7) (Britten et al., 1970; McConaughy & McCarthy, 1972) both at 100 °C. The temperatures of the column bed, the circulating bath, and the elution buffer were continuously monitored; reported temperatures of elution reflect those directly measured in the column bed.

Thermal elution profiles were displayed by plotting the cumulative percent of bound cpm eluted at each temperature increment (including the 0.5 M NaPB and 8.0 M urea wash fractions at 100 °C) vs. temperature. Material removed from the column at 55 °C (less than 3% of the total cpm applied initially) was not included in the thermal elution profile. Bound chromatin DNA and pure DNA were quantitatively eluted. Analysis was performed with a Beckman LS-230 liquid scintillation spectrometer and scintillant composed of a 2:1 mixture of reagent grade toluene and scintillation grade Triton X-100 containing Omnifluor (New England Nuclear). Aqueous samples were counted in 3.0 mL of 0.12 M NaPB and 16 mL of scintillant after at least 12 h in the dark. Tritium efficiency under these conditions was 30.0%.

Optically Monitored Thermal Denaturation of Chromatin and DNA. Sheared chromatin and DNA samples were denatured in various buffers in a thermal cuvette controlled by a Gilford Model 2527 Thermo-Programmer. Hyperchromicity

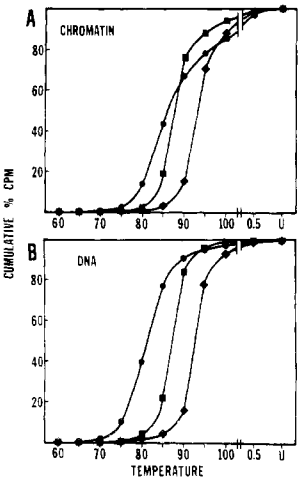

FIGURE 1: (A) Hydroxylapatite thermal elution profiles of cultured muscle chromatin, comparing 0.12 M NaPB (♦), 0.12 M KPB (■), and 0.025 M CsPB (●) elution buffer systems. Chromatin was prepared and sheared at 15 000 psi in a French pressure cell. Chromatin (containing approximately 15 μg of DNA at 200 000 cpm/μg) was loaded onto a 2.5-mL bed volume hydroxylapatite column; unbound material was removed at 55 °C and thermal chromatography was performed by increasing the temperature to 100 °C in 5 °C increments and eluting melted material at each increment. After the elution buffer rinse at 100 °C, remaining bound material was removed from the column with 0.5 M NaPB (0.5) and then 8.0 M urea, 0.24 M NaPB, 0.01 M EDTA (U). Data are plotted as cumulative percent cpm eluted vs. temperature. T_m's are 92.5, 87.5, and 86.5 °C for 0.12 M NaPB, 0.12 M KPB, and 0.025 M CsPB, respectively. (B) Hydroxylapatite thermal elution profiles of DNA purified from the corresponding 15 000 psi sheared chromatin preparations of A comparing 0.12 M NaPB (♦), 0.12 M KPB (■), and 0.025 M CsPB (●) elution buffer systems. Samples, containing approximately 15 μg of DNA, were applied to hydroxylapatite columns at 55 °C and thermally chromatographed as in A. T_m's are 92.5, 87.5, and 81.5 °C for 0.12 M NaPB, 0.12 M KPB, and 0.025 M CsPB, respectively.

and temperature were monitored with a Gilford Model 2400-2 automatic recording spectrophotometer. Experiments were begun at 50 °C and temperature was increased at 0.5 °C/min until a plateau in hyperchromicity was reached. Data are expressed as cumulative percent hyperchromicity vs. temperature.

DNA Sizing Methods. DNA fragments purified from chromatin sheared at different pressures were sized by gel electrophoresis by a modified method of Sharp et al. (1973) and by electron microscopy by a modified technique of Kleinschmidt (1968). Gels consisted of 1% agarose (w/v) in 0.04 M Tris (pH 7.9), 0.005 M sodium acetate, 0.001 M EDTA (pH 7.7) with a 2% agarose spacer. Samples, in 15% sucrose, were loaded onto the gel with λ DNA (Miles Laboratories) restriction fragments (in 15% sucrose and 0.025% bromphenol blue) produced by digestion with *Hin*dIII restriction enzyme (Miles Laboratories) isolated from *H. influenza*. Gels were run at 40 mA for 3 h and stained in 0.5 μg/mL ethidium bromide in the above buffer. Fluorescence was visualized under UV illumination.

Samples were prepared for electron microscopy by lowering a spreading solution (60 μL of DNA at 5 μg/mL in 0.15 M NaPB, 20 μL of cytochrome *c* at 0.1 μg/mL, 10 μL of 0.1 M Tris, 0.01 M EDTA, pH 8.5) onto a distilled water hypophase. Samples were placed on 150 mesh pallodian coated, carbon stabilized copper grids and stained in phosphotungstic acid and uranyl acetate for 15 and 30 s, respectively. After rotary shadowing with platinum–palladium, grids were examined in an RCA-3H EMU at 100 kV and random areas were photographed. Bacteriophage PM_2 DNA (6.4×10^6 daltons, Espejo et al., 1969) was included as a molecular size standard.

Results

Comparative Characteristics of Sodium, Potassium, and Cesium Elution Buffers. Chromatin and purified DNA were fractionated on hydroxylapatite using the conventional 0.12 M NaPB elution buffer, as illustrated in Figures 1A and 1B. In this buffer, *both* chromatin and DNA eluted within a narrow temperature range with a T_m of 92 to 93 °C and, thus, did not display the differences seen in low ionic strength solution denaturation. The high T_m's in 0.12 M NaPB were due, in part, to the stabilizing effects of the high cation concentration (Marmur & Doty, 1962).

These studies were repeated using 0.12 M KPB, which was expected to yield a lower hydroxylapatite T_m for both DNA and chromatin (compared with 0.12 M NaPB) because of its greater eluting power (Bernardi, 1971; Martinson, 1973a,b). As illustrated in Figures 1A and 1B, both chromatin and DNA eluted within a narrow temperature range at T_m's of 87–88 °C. These data are similar to 0.12 M KPB elution profiles obtained by David et al. (1974) using other chromatin and DNA sources.

These studies were again repeated using CsPB elution buffer. The greater eluting power of Cs^+ ion (Martinson, 1973a,b) allows adequate strand discrimination at a lower ionic concentration than is possible with either NaPB or KPB. The resulting decreased cation stabilization of duplex structures might therefore reveal, by hydroxylapatite thermal elution, the differences in chromatin and DNA denaturation behavior seen in low ionic strength solution. Initial experiments employed 0.01 M CsPB as used by others (McCarthy et al., 1973; Nasser & McCarthy, 1975). However, in our hands, this concentration was clearly unable to displace single-stranded chromatin from hydroxylapatite; at 100 °C, more than 65% of the initially applied chromatin DNA remained bound to hydroxylapatite. When purified single-stranded DNA was applied to hydroxylapatite at 65 °C, less than 20% was eluted by 0.01 M CsPB.

CsPB at 0.025 M was empirically determined to have adequate strand discrimination properties under the conditions employed for chromatin and DNA hydroxylapatite fractionation. Concentrations above 0.03 M displaced bound duplex structures, while concentrations below 0.02 M failed to quantitatively elute single-stranded material. CsPB at 0.025 M had strand discrimination properties similar to 0.12 M NaPB and was employed in subsequent experiments.

The hydroxylapatite thermal elution of chromatin and DNA in 0.025 M CsPB is shown in Figures 1A and 1B. In 0.025 M CsPB, chromatin had a T_m of 86.5 °C and eluted over a wider range than in either 0.12 M NaPB or KBP; DNA displayed a 5 °C lower T_m and a significantly narrower range of denaturation than chromatin (Table I). These are features characteristic of thermal denaturation of chromatin and DNA in solution and were not observed using either 0.12 M KPB or 0.12 M NaPB.

Comparative Features of Hydroxylapatite and Solution Denaturation Profiles. The hydroxylapatite–nucleic acid system is complex and involves interactions not present when chromatin or DNA denatures in solution. The hydroxylapatite elution profile of DNA in 0.025 M CsPB was compared with the denaturation profile of DNA in 0.025 M CsPB solution as shown in Figure 2. A similar comparison using chromatin was not possible due to the aggregation of chromatin above 65 °C in this higher ionic strength buffer. (Chromatin aggregation in 0.025 M CsPB occurring above 65 °C was presumably prevented during hydroxylapatite thermal chromatography by the physical immobilization of chromatin fragments on hydroxylapatite at 55 °C.) Denaturation profiles of DNA in

we have been conducting labeling experiments to determine which of the components in complex III are at the matrix side and which are exposed at the intracistral side of the mitochondrial inner membrane. The cross-linking and labeling data taken together can be used to develop a model of the arrangement of polypeptides in complex III. This model will be presented along with the labeling experiments in a forthcoming paper.

Acknowledgments

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References

Anderson, G. W., Zimmerman, J. E., and Callahan, F. M. (1964), *J. Am. Chem. Soc. 89,* 1839.
Baird, B. A., and Hammes, G. G. (1976), *J. Biol. Chem. 251,* 6953.
Bell, R. L., and Capaldi, R. A. (1976), *Biochemistry 15,* 996.
Birchmeier, W., Kohler, C. E., and Schatz, G. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 4334.
Bragg, P. D., and Hou, C. (1975), *Arch. Biochem. Biophys. 167,* 311.
Briggs, M. M., and Capaldi, R. A. (1977), *Biochemistry 16,* 73.
Capaldi, R. A., Bell, R. L., and Branchek, T. (1977), *Biochem. Biophys. Res. Commun. 74,* 425.
Coggins, J. R., Hopper, E. A., and Perham, R. N. (1976), *Biochemistry 15,* 2527.
Das Gupta, V. D., and Rieske, J. S. (1973), *Biochem. Biophys. Res. Commun. 54,* 1247.
Gellerfors, P., and Nelson, B. S. (1975), *Eur. J. Biochem. 52,* 433.
Hare, J. F., and Crane, F. L. (1974), *Sub-Cell. Biochem. 3,* 1.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 93,* 265.
Lutter, L. C., Orlanderl, F., and Fasold, H. (1974), *FEBS Lett. 48,* 288.
Rieske, J. S. (1967), *Methods. Enzymol. 10,* 239.
Rieske, J. S. (1967), *Biochim. Biophys. Acta 456,* 195.
Robinson, N. C., and Capaldi, R. A. (1977), *Biochemistry 16,* 375.
Smith, R. J., and Capaldi, R. A. (1977), *Biochemistry 16,* 2629.
Swank, R. T., and Munkres, K. D. (1971), *Anal. Biochem. 39,* 462.
Wang, K., and Richards, F. M. (1974), *J. Biol. Chem. 249,* 8005.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244,* 4406.
Whittmann, H.-G. (1976), *Eur. J. Biochem. 61,* 1.
Williams, J. R. (1964), *Arch. Biochem. Biophys. 107,* 537.
Yu, C. A., Yu, L., and King, T. E. (1974), *J. Biol. Chem. 249,* 4905.

Cytochrome b_{562} from *Escherichia coli*: Conformational, Configurational, and Spin-State Characterization†

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ABSTRACT: The protein conformation, heme configuration, and the spin state of heme iron of cytochrome b_{562} from *Escherichia coli* have been investigated using circular dichroism (CD), optical, and resonance Raman (RR) spectroscopy as the probes. Studies are reported on the effect of ionic strength on the CD spectra of the ferric and ferrous forms, of temperature variation on the CD spectrum of the ferric form, and of pH variation in the range 3–11 on optical, circular dichroism, and resonance Raman spectra of the ferric form of the protein. Differences in conformational sensitivity to increasing ionic strength of the medium are seen between ferri- and ferrocytochrome b_{562}. The thermal denaturation of ferricytochrome b_{562} at neutral pH is found to occur in two distinct steps centered at about 35 and 67 °C, with $\Delta H°$ and $\Delta S°$ of about 74 eu and 23 kcal, and 254 eu and 74 kcal, respectively. The effect of pH on the optical spectrum of ferricytochrome b_{562} is seen in the presence of three distinct pH forms in the range 3–11 with apparent pKs of about 6 and 8.7. The acidic transition is accompanied by minimal perturbation of the optical spectrum. The basic transition, pK_a of 8.7, is accompanied by a red shift of the Soret peak and the visible spectrum and the generation of a new band at about 635 nm. The CD spectrum does not indicate any significant variation of the protein secondary structure in any of the pH transitions, but the heme symmetry is altered during the alkaline transition from a less symmetric heme to a more symmetric heme. The resonance Raman spectra of the three pH forms are found to be typical of low-spin heme iron systems. A heme configuration of methionine/histidine ligation at the two axial positions of heme iron for the acidic and neutral forms and transformation to a form with lower heme symmetry, i.e., with lysine/histidine or histidine/histidine ligation, have been concluded.

Cytochrome b_{562} from *E. coli* is thought to be a soluble electron carrier for a system located in the membrane, although the protein itself is not bound to the membrane (Lemberg & Barrett, 1973; Hager & Itagaki, 1967). In mammalian systems this physiological function is attributed to cytochrome *c*, while cytochromes of type *b* are membrane-localized (Dickerson & Timkovich, 1975; Hagihara et al., 1975; Ferfuson-Miller et al., 1978). The similarity in function of the two quite different proteins has attracted our attention, and, in order to understand this phenomenon, we have undertaken an extensive physico-

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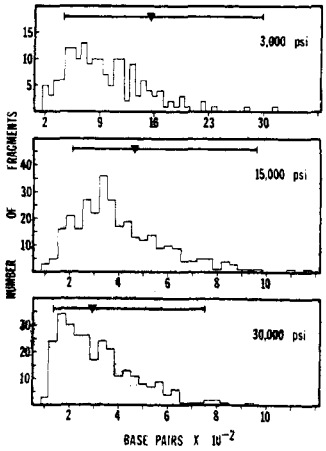

FIGURE 4: Fragment size distribution of DNA purified from unlabeled cultured muscle chromatin. Chromatin was sheared in 0.025 M CsPB at 30 000, 15 000, and 3000 psi in a French pressure cell. DNA was purified from chromatin and analyzed by electrophoresis on 1% agarose slab gels using *Hin*dIII restriction nuclease fragments of λ bacteriophage DNA as size standards and by electron microscopy using bacteriophage PM_2 DNA as a size standard. Fragment measurements from electron micrographs were converted to base pair lengths using the PM_2 DNA standard and are plotted as a fragment size histogram. The median length, number average mean length, length average mean length all in base pairs, and the sample size are: 820, 948, 1210, and 172 for 3000 psi sheared fragments; 360, 400, 486, and 307 for 15 000 psi sheared fragments; and 270, 317, 394, and 283 for 30 000 psi sheared fragments. Gel electrophoresis data are displayed as a range of migration expressed in terms of base pair lengths as determined from λ restriction fragment standards. The position of the maximum fluorescence is noted by an arrowhead and corresponds to base pair lengths of 1550, 450, and 290 for fragments sheared at 3000, 15 000, and 30 000 psi, respectively.

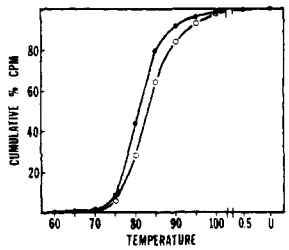

FIGURE 5: The hydroxylapatite thermal chromatography of a mixture of [^{3}H]thymidine-labeled DNA purified from 15 000 psi sheared cultured muscle chromatin and total histones from day 16 embryonic chick leg and thigh muscle tissue. Four micrograms of both DNA and histones were mixed in 1.0 mL of 0.025 M CsPB at 4 °C and immediately applied to a hydroxylapatite column at 55 °C. The mixture was thermally chromatographed and its profile compared to a thermal elution profile of DNA alone run in a parallel column. T_m's are 81.0 and 83.0 °C for the DNA (●) and the DNA–histone mixture (○), respectively.

Studies of Chromatin Proteins and Protein–DNA Interactions during Hydroxylapatite Thermal Elution. Some aspects concerning chromatin proteins and DNA–protein interactions during hydroxylapatite thermal chromatography were investigated using 0.025 M CsPB. The ability of the CsPB–hydroxylapatite system to detect protein stabilization of DNA in dilute mixtures of sheared DNA and homologous total histones is shown in Figure 5. Such mixtures produce chromatin-like material as assayed by circular dichroism spectroscopy (Ordahl et al., 1978). Figure 5 demonstrates a highly reproducible 2 °C difference in T_m when the DNA–histone mixture is compared by thermal chromatography to DNA alone.

The fate of proteins during chromatin fractionation was studied by labeling histones with [^{3}H]lysine and nonhistones with [^{3}H]tryptophan during the myoblast proliferation phase

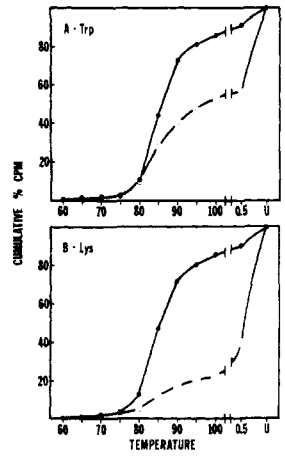

FIGURE 6: The hydroxylapatite thermal chromatography of double-labeled 15 000 psi sheared muscle culture chromatin. Chromatin was labeled with 0.065 μCi/mL [^{14}C]thymidine and 5 μCi/mL [^{3}H]tryptophan (A) or [^{3}H]lysine (B) during the myoblast proliferation phase. Thermal chromatography was carried out in 0.025 M CsPB as described. Data, corrected for ^{14}C spillover into the ^{3}H channel, are expressed as the cumulative percent of total eluted cpm eluting vs. temperature, ^{14}C (●), ^{3}H (○).

in separate muscle cultures. DNA was labeled with [^{14}C]-thymidine in both groups. The majority of the [^{3}H]lysine incorporation in chromatin should be found in the lysine rich histones H1, H2B, and H2A, while tryptophan incorporation occurs exclusively in the nonhistone proteins (Hnilica, 1975). Figure 6 illustrates the double-labeled chromatin elution profiles and reveals that much protein remained bound to the column. Whereas DNA was quantitatively eluted, 54% of the initially applied [^{3}H]lysine labeled material and 45% of the initially applied [^{3}H]tryptophan labeled material remained bound to the column even after washing with 0.5 M NaPB and 8.0 M urea at 100 °C. Of the total cpm eluted, approximately 60% of the [^{3}H]lysine-labeled material and 45% of the [^{3}H]-tryptophan-labeled material appeared in the 8.0 M urea wash fractions. Furthermore, while less than 3% of the initially applied DNA cpm was removed at 55 °C, 11% of the total [^{3}H]lysine-labeled material and 16% of the [^{3}H]tryptophan labeled material appeared in the unbound fraction at 55 °C. This indicated that either some labeled, nonbinding, nonchromosomal protein material had been isolated with chromatin or that true chromosomal protein was displaced from chromatin and eluted at 55 °C.

A final experiment was performed to determine whether protein remaining on hydroxylapatite affects the denaturation and elution of DNA during thermal chromatography. Labeled DNA was applied to a column; then an equal amount of freshly prepared, unlabeled chromatin was added to the column and thermal elution was carried out in 0.025 M CsPB. Figure 7 compares the resulting profile with one from purified DNA alone in a parallel column. Except for small differences at the highest temperatures, the profiles are superimposable suggesting that free protein generated from chromatin does not affect the thermal stability of hydroxylapatite binding of free DNA (and presumably chromatin) remaining on the column. Such free protein probably occupies other binding sites on hydroxylapatite.

Discussion

Hydroxylapatite thermal chromatography is designed to separate portions of the genome varying in thermal stability due to qualitative or quantitative differences in protein–DNA interactions. Such protein–DNA interactions have been

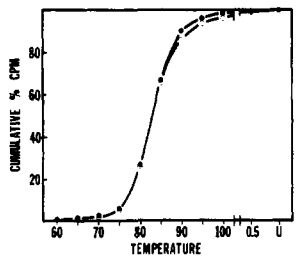

FIGURE 7: The hydroxylapatite thermal chromatography of [^{3}H]thymidine-labeled DNA in the presence of unlabeled, 15 000 psi sheared chromatin (○). Labeled DNA, obtained from 15 000 psi sheared cultured muscle chromatin, was applied to a hydroxylapatite column and unbound material was removed. Then, a sample of freshly prepared, unlabeled, 15 000 psi sheared cultured muscle chromatin containing an approximately equal amount of DNA was applied to the column and thermal chromatography was carried out. A parallel column to which only labeled DNA was applied was similarly run (●). Data are expressed as the cumulative percent of the total cpm eluting vs. temperature. T_m's are 82.0 °C for both profiles.

demonstrated by comparing denaturation features of DNA and chromatin in solution (Huang & Bonner, 1962; Marushige & Ozaki, 1967; Paoletti & Huang, 1969; Li & Bonner, 1971). However, the use of hydroxylapatite, required as a means for actual separation of chromatin fragments of differential thermal stability, imposes rather severe constraints on the conditions of denaturation that may be employed. Specifically, only certain buffers at certain concentrations meet the required strand discrimination criteria.

Using the conventional 0.12 M NaPB (also used by McConaughy & McCarthy, 1972), both chromatin and DNA exhibited virtually identical hydroxylapatite elution profiles and did not reflect the major differences in T_m and temperature range of denaturation observed in low ionic strength solution. Similar results were obtained with 0.12 M KPB. Two factors probably account for this observation: nucleic acid duplex stabilization by (1) binding to the hydroxylapatite crystal surface and by (2) cation neutralization of the DNA backbone combined to reduce the protein stabilization of chromatin DNA to secondary importance. Furthermore, the high cation concentration (0.18 M) of both NaPB and KPB at elevated temperatures likely promotes the removal or rearrangement of chromatin proteins prior to actual DNA duplex denaturation (Jensen & Chalkley, 1968; Clark & Felsenfeld, 1971).

CsPB, because of its greater eluting power, has adequate strand discrimination on hydroxylapatite at far lower concentrations than either KPB or NaPB. The combination of decreased nucleic acid-hydroxylaptite binding and decreased cation stabilization of duplex structure resulted in chromatin and DNA elution profiles similar to those seen in thermal denaturation in low ionic strength media. Thus, of the buffers examined, only CsPB revealed the protein mediated thermal stabilization of chromatin DNA.

As previously mentioned, nucleic acid denaturation in solution is essentially independent of fragment size; a hyperchromic shift is detected even when only a portion of an individual fragment melts. Hydroxylapatite thermal elution of nucleic acids is, however, affected by fragment size. As shown in Figures 3A and 3B, increasing fragment size leads to an increased T_m. The greater effects of fragment size on chromatin compared with DNA may be explained by considering the factors which lead to regional heterogeneity in thermal stability in a given fragment of DNA and chromatin. The temperature of elution of a purified DNA fragment depends on base composition; a G + C rich region will cause the entire fragment to remain bound to hydroxylapatite even though less G + C rich regions have denatured. Differences in regional heterogeneity in base composition sufficient to cause a large shift in elution profile are probably unlikely in DNA from chromatin sheared at 30 000 psi and 15 000 psi which had number average mean lengths of 320 and 400 base pairs, respectively. However, DNA from chromatin sheared at 3000 psi averaged 950 base pairs and thus would be more likely to exhibit a higher T_m than smaller sized DNA populations. In addition to base compositional heterogeneity, a given fragment of chromatin might also have regions of differential thermal stability caused by qualitative or quantitative differences in DNA-protein interactions. These differences might account for the T_m shift observed between 30 000 and 15 000 psi sheared preparations as well as the 3000 psi sheared material.

Using 0.025 M CsPB, the hydroxylapatite thermal elution system was able to detect protein mediated thermal stability of DNA in in vitro nucleohistone preparations. Yet, DNA bound to the column was unaffected by free protein generated during chromatin thermal elution even though significant amounts of protein remain on the column throughout the elution procedure. In conjunction with the other experiments described, these data indicate that, under the conditions employed, fragments of chromatin or DNA can be segregated on the basis of differential thermal stability by hydroxylapatite chromatography.

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References

Anderson, K. M., Chance, H., & Kadohama, N. (1975) *Exp. Cell Res. 94,* 176-190.

Arnold, E. A., & Young, K. E. (1974) *Arch. Biochem. Biophys. 164,* 73-89.

Beer, M., & Thomas, C. A. (1961) *J. Mol. Biol. 3,* 699-700.

Berkowitz, E. M., & Doty, P. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72,* 3328-3332.

Bernardi, G. (1971) *Methods Enzymol. 21D,* 95-147.

Billing, R. J., & Bonner, J. (1972) *Biochim. Biophys. Acta 281,* 452-462.

Birns, K. I., & Thomas, C. A. (1961) *J. Mol. Biol. 3,* 289-300.

Bonner, J., Chalkley, G. R., Dahmus, M., Fambrough, D., Fujimura, F., Huang, R. C. C., Huberman, J., Jensen, R., Marushige, K., Ohlenbusch, H., Livera, B., & Widholm, J. (1968) *Methods Enzymol. 12B,* 3-65.

Bonner, J., Garrard, W. T., Gottesfeld, J., Holmes, D. S., Sevall, J. S., & Wilkes, M. (1973) *Cold Spring Harbor Symp. Quant. Biol. 38,* 303-310.

Britten, R. J., Pavich, M., & Smith, J. (1970) *Carnegie Inst. Washington, Yearb. 68,* 400-402.

Burkholder, G. D., & Weaver, M. G. (1975) *Exp. Cell Res. 92,* 518-522.

Caplan, A. I. (1976) *J. Embryol. Exp. Morphol. 36,* 175-181.

Chalkley, R., & Jensen, R. H. (1968) *Biochemistry 7,* 4380-4388.

Charles, M. A., Ryffel, G. U., Obinata, M., McCarthy, B. J., & Baxter, J. D. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72,*

1787-1791.
Chesterton, C. J., Coupar, B. E. H., & Butterworth, P. H. W. (1974) *Biochem. J. 143,* 73-81.
Clark, R. J., & Felsenfeld, G. (1971) *Nature (London) New Biol. 229,* 101-106.
David, J., Gordon, J. S., & Rutter, W. J. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71,* 2808-2812.
Duerksen, J. D., & McCarthy, B. J. (1971) *Biochemistry 10,* 1471-1478.
Espejo, R. T., Canelo, E. S., & Sinsheimar, R. C. (1969) *Proc. Natl. Acad. Sci. U.S.A. 63,* 1164-1168.
Frenster, J. H. (1965) *Nature (London) 206,* 680-683.
Frenster, J. H., Allfrey, V. G., & Mirsky, A. E. (1963) *Proc. Natl. Acad. Sci. U.S.A. 50,* 1026-1032.
Gottesfeld, J., Garrard, W. T., Bagi, G., Wilson, R. F., & Bonner, J. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71,* 2193-2197.
Gottesfeld, J., Murphy, R. F., & Bonner, J. (1975) *Proc. Natl. Acad. Sci. U.S.A. 11,* 4404-4408.
Gottesfeld, J., Bagi, G., Berg, B., & Bonner, J. (1976) *Biochemistry 15,* 2472-2483.
Herrmann, H., Heywood, S. M., & Marchok, A. C. (1970) *Curr. Top. Dev. Biol. 5,* 181-243.
Hnilica, L. S. (1975) *Methods Enzymol. 40E,* 102-138.
Howk, R. S., Anisowica, A., Silverman, A. Y., Parks, W. P., & Scolnick, E. M. (1975) *Cell 4,* 321-327.
Huang, R. C. C., & Bonner, J. (1962) *Proc. Natl. Acad. Sci. U.S.A. 48,* 1216-1222.
Jalouzot, R., Gontcharoff, M., & Briane, D. (1975) *FEBS Lett. 59,* 221-224.
Janowski, M., Nasser, D. S., & McCarthy, B. J. (1972) *Acta Endocrinol., Suppl. 168,* 112-119.
Jensen, R. H., & Chalkley, R. (1968) *Biochemistry 7* 4388-4395.
Keller, R. K., Socher, S. H., Krall, J. F., Chandra, T., & O'Malley, B. W. (1975) *Biochem. Biophys. Res. Commun. 66,* 453-459.
Kleinschmidt, A. K. (1968) *Methods Enzymol. 12B,* 361-377.
Kreig, P., & Wells, J. R. E. (1976) *Biochemistry 15,* 4549-4558.
Levner, M. H., Fromson, D., Rickles, S., Graham, M., & Nemer, M. (1975) *Arch. Biochem. Biophys. 169,* 638-649.
Li, H. J., & Bonner, J. (1971) *Biochemistry 10,* 1461-1470.
Markov, G. G., Ivanov, I. G., & Pashev, I. G. (1975) *J. Mol. Biol. 93,* 405-413.
Marmur, J., & Doty, P. (1962) *J. Mol. Biol. 5,* 109-118.
Martinson, H. G. (1973a) *Biochemistry 12,* 139-145.
Martinson, H. G. (1973b) *Biochemistry 12,* 145-150.
Marushige, K., & Bonner, J. (1966) *J. Mol. Biol. 15,* 160-174.
Marushige, K., & Bonner, J. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68,* 2941-2944.
Marushige, K., & Ozaki, H. (1967) *Dev. Biol. 16,* 474-488.
McCarthy, B. J., Nishiura, J. T., Doenecke, D., Nasser, D. S., & Johnson, C. B. (1973) *Cold Spring Harbor Symp. Quant. Biol. 38,* 763-771.
McConaughy, B. L., & McCarthy, B. J. (1972) *Biochemistry 11,* 998-1003.
Miyazawa, Y., & Thomas, C. A. (1965) *J. Mol. Biol. 11,* 223-237.
Murphy, E. C., Hall, S. H., Shepherd, J. H., & Weiser, R. S. (1973) *Biochemistry 12,* 3843-3853.
Nasser, D. S., & McCarthy, B. J. (1975) *Methods Enzymol. 40E,* 93-97.
Ordahl, C. P., Johnson, T. R., & Caplan, A. I. (1978) submitted for publication.
Paoletti, R. A., & Huang, R. C. C. (1969) *Biochemistry 8,* 1615-1625.
Paul, I. J., & Duerksen, J. D. (1976) *Biochem. Biophys. Res. Commun. 68,* 97-105.
Pays, E., & Flamand, J. (1976) *FEBS Lett. 61,* 166-170.
Pederson, T., & Bhorjee, J. S. (1975) *Biochemistry 14,* 3238-3242.
Polacow, I., & Simpson, R. T. (1973) *Biochem. Biophys. Res. Commun. 52,* 202-207.
Reeck, G. R., Simpson, R. T., & Sober, H. A. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69,* 2317-2321.
Reeck, G. R., Simpson, R. T., & Sober, H. A. (1974) *Eur. J. Biochem. 49,* 407-414.
Sharp, P. A., Sugden, B., & Sambrook, J. (1973) *Biochemistry 12,* 3055-3063.
Simpson, R. T. (1975) *Biochem. Biophys. Res. Commun. 65,* 552-558.
Simpson, R. T., & Polacow, I. (1973) *Biochem. Biophys. Res. Commun. 55,* 1078-1084.
Simpson, R. T., & Reeck, G. R. (1973) *Biochemistry 12,* 3853-3858.
Wilson, D. A., & Thomas, C. A. (1973) *Biochim. Biophys. Acta 331,* 333-340.
Yasmineh, W. G., & Yunis, J. J. (1969) *Biochem. Biophys. Res. Commun. 35,* 779-782.
Yunis, J. J., & Yasmineh, W. G. (1970) *Science 168,* 263-265.