Since the data presented above show decreasing protein and mRNA abundances in the same order, the "transcriptional regulation" observed here may merely reflect a probability of "fall-off" of each transcriptase molecule as it proceeds along its template beyond each gene.

References

Bonner, W. M., and Laskey, R. A. (1974), *Eur. J. Biochem. 46,* 83.
Both, G. W., Moyer, S. A., and Banerjee, A. K. (1975), *J. Virol. 15,* 1012.
Breindl, M., and Holland, J. J. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 2545.
Grubmann, N. J., Moyer, S. A., Banerjee, A. K., and Ehrenfeld, E. (1975), *Biochem. Biophys. Res. Commun. 62,* 531.
Kang, C. Y., and Prevec, L. (1971), *Virology 46,* 678.
Knipe, D., Rose, J. K., and Lodish, H. F. (1975), *J. Virol. 15,* 1004.
Laemmli, U. K. (1970), *Nature (London) 227,* 680.
Laskey, R. A., and Mills, A. D. (1975), *Eur. J. Biochem. 56,* 335.
McConkey, E. H., and Hopkins, J. W. (1969), *J. Mol. Biol. 39,* 545.
Morrison, T., Stampfer, M., Baltimore, D., and Lodish, H. F. (1974), *J. Virol. 13,* 62.
Mudd, J. A., and Summers, D. F. (1970), *Virology 42,* 328.
Obijeski, J. F., Marchenko, A. T., Bishop, D. H. L., Cann, B. W., and Murphy, F. A. (1974), *J. Gen. Virol. 22,* 21.
Pinder, J. C., Staynov, D. Z., and Gratzer, W. B. (1974), *Biochemistry 13,* 5373.
Rose, J. K., and Knipe, D. (1975), *J. Virol. 15,* 994.
Roy, P., and Bishop, D. H. L. (1972), *J. Virol. 9,* 946.
Stamminger, G., and Lazzarini, R. A. (1974), *Cell 3,* 85.
Stanley, W. M., Jr., and Bock, R. M. (1965), *Biochemistry 4,* 1302.
Wagner, R. R. (1975), in *Compr. Virol. 4,* 1.
Wagner, R. R., Kiley, M. P., Snyder, R. M., and Schnaitmann, C. A., (1972), *J. Virol. 9,* 672.

Interactions of Mercury and Copper with Constitutive Heterochromatin and Euchromatin in Vivo and in Vitro†

Sara E. Bryan,* Sam J. Simons, Douglas L. Vizard,‡ and Kenneth J. Hardy‡

ABSTRACT: Mouse liver nuclei were fractionated into (condensed) heterochromatin and (noncondensed) euchromatin by differential centrifugation of sonicated nuclei. The fractions were subsequently characterized as unique nuclear species by thermal denaturation derivative profile analysis, which revealed the heterochromatin fraction enriched in satellite DNA and by endogenous metal content, which displayed partitioning of mercury in euchromatin over heterochromatin by a 10:1 ratio, with a comparatively uniform distribution of copper in both fractions. Fractionation of nuclei following in vivo challenge with copper showed enrichment of copper in heterochromatin, relative to euchromatin, while in vivo exposure to mercury resulted in a 20-fold accumulation of mercury in euchromatin, relative to heterochromatin. Using gel filtration and equilibrium dialysis to measure in vitro binding under relatively physiologic conditions of pH (6.0–7.0) and ionic strength (standard saline citrate or saline), the condensed and noncondensed chromatin fractions exhibited binding specificities toward mercury and copper similar to that observed in the in vivo metal challenge experiments. The level of mercury which binds to euchromatin in vitro, when measured either in physiologic [standard saline citrate (SSC)] or in dilute (1:100 SSC) salt solutions, was comparable (approximately 3 μg of Hg/mg of DNA) to that of in vivo euchromatin-bound mercury after 1 month of challenge with dietary metal. In contrast, copper showed little or no preference for the nuclear fractions in dilute salt solutions and displayed patterns which mimic in vivo binding only at higher ionic strengths (saline). Removal of proteins from the chromatin fractions resulted in a loss of binding specificity toward both metals. Therefore, the binding selectivity of condensed and noncondensed chromatin toward both mercury and copper appears to arise from protein or from protein–DNA associations. The state of chromatin condensation is especially critical in the case of copper.

A growing body of evidence supports the view that controlling elements which regulate the activity of genes in eukaryotic cells reside in chromatin, the complex interphase chromosomal material (Stein et al., 1974; Stein and Farber, 1972; Gilmour, 1974; Wilhelm et al., 1971). The evidence implicates chromosomal proteins and, in particular, nonhistone proteins as the regulatory elements (Stein et al., 1974; Gilmour, 1974; and Spelsberg et al., 1972). However, the fact that chromatin contains metals (along with DNA, RNA, and histone and nonhistone protein) which display unique binding affinities for each of the nuclear constituents could be important in understanding the structure of the genome and how chromosomal proteins regulate its function.

† From the Department of Biological Sciences, University of New Orleans (S.E.B. and S.J.S.), New Orleans, Louisiana, and the Department of Biochemistry and Biophysics (D.L.V. and K.J.H.), M.D. Anderson Hospital and Tumor Institute, University of Texas, Houston, Texas. *Received June 18, 1975.* This investigation was supported by a NIH research Grant 1 R01 ES00802-0 from National Institute of Environmental Health Sciences.

‡ Present address: Department of Biochemistry and Biophysics, M.D. Anderson Hospital and Tumor Institute, University of Texas, Houston, Texas.

Certain divalent metals such as Ca^{2+} appear to be crucial in maintaining the structure of nucleoprotein complexes (Wagner and Vandergrift, 1972). Others have been utilized as effective chemical agents to probe chromatin structure. For example, manganese coupled with the nuclease reaction has been used to demonstrate accessibility of DNA binding sites (Clark and Felsenfeld, 1974). Similarly, magnesium can be employed to fractionate chromatin to reveal the heterogeneity of the nuclear material (Arnold and Young, 1974). Both copper (Zimmer et al., 1971; Bryan and Frieden, 1967) and mercury (Nandi et al., 1965) are known to interact with DNA (in vitro) in a specific manner. Although these metals are among the naturally occurring metal constituents of the nucleus (Bryan et al., 1974a; Hardy and Bryan, 1975), very little is known about how they interact with chromatin or what function, if any, they play in the metabolism of the nucleus.

Chromatin can be fractionated into unique species of (condensed) heterochromatin and (noncondensed) euchromatin by techniques which preserve the native state of the materials (Frenster, 1969; Yasmineh and Yunis, 1970). Such a fractionation has been used with success to demonstrate the localization of highly redundant satellite DNA sequences in condensed chromatin (Yasmineh and Yunis, 1970; Yunis and Yasmineh, 1970; Duerksen and McCarthy, 1971) and to separate fractions which differ in their ability to act as template for the synthesis of RNA in vitro (Frenster et al., 1963; Frenster, 1969). We have employed the same fractionation scheme to demonstrate different patterns of metal localization and accumulation for mercury (Bryan et al., 1974b) and for copper (Hardy and Bryan, 1975). In this paper, we examine the binding of copper and mercury with condensed and noncondensed chromatin in more detail by using in vitro systems which mimic in vivo physiological conditions of pH, ionic strength, and metal concentration. The parameters of thermal denaturation, endogenous metal content, protein:DNA ratios, and uv absorbance are used to characterize the chromatin fractions prior to metal binding by gel filtration and equilibrium dialysis. The results are compared with in vivo binding data.

Materials and Methods

Materials

Male Swiss Webster strain mice obtained commercially were 1 to 2 months old at the beginning of each experiment. Animals were fed Purina Rat Chow ad libitum throughout the 1–2 month study and given either deionized drinking water (control) or deionized drinking water containing varying levels of cupric chloride or of mercuric chloride (experimental) as previously described (Bryan et al., 1974a; Hardy and Bryan, 1975). At the end of a given time period, animals were ether anesthetized and exsanguinated by cardiac puncture. Livers were removed and placed in physiological saline at 4 °C for immediate use. A typical experiment consisted of approximately 40 g of liver tissue.

Chemicals

Ultrapure Tris and Ultrapure sucrose were obtained from Schwarz/Mann. Phenylmethanesulfonyl fluoride (PMSF[1]) and dimethyl sulfoxide (Me_2SO) were obtained from Sigma Chemical Company. Other chemicals were reagent grade. Deionized, chelexed (100) water was used in cleaning glassware and in the preparation of solutions.

Methods

Isolation and Fractionation of Nuclei. A modification of the technique of Blobel and Potter (Blobel and Potter, 1966) was used to prepare mouse liver nuclei. Fresh liver tissue was washed and minced in cold physiologic saline, blotted dry, and weighed. A 20% (w/v) homogenate was prepared in solution consisting of 0.5% Triton X-100, 0.25 M sucrose, TMKC buffer (0.05 M Tris, 5.0 mM $MgCl_2$, 3.0 mM KCl, 0.2 mM $CaCl_2$, pH 7.4), 1% Me_2SO, and 0.5 mM PMSF. Tissue disruption was effected utilizing a motor-driven Potter-Elvehjen homogenizer for 15 strokes. After filtering through four layers of cheesecloth, the homogenate was centrifuged at 600*g* in a Sorvall RC-2 centrifuge at 4 °C for 15 min. The pellet was rehomogenized with a loose fitting, glass-glass dounce homogenizer for four strokes using the same buffer. After recentrifugation at 600*g*, the pellet was suspended in 2.2 M sucrose–TMKC, pH 7.4, overlaid on 2.3 M sucrose–TMKC, pH 7.4, and spun at 100 000*g* for 1 h. Purified nuclei so obtained were stored at −20 °C in 2.2 M sucrose–TMKC, pH 7.4, overnight prior to use. At this sucrose concentration, nuclei do not freeze at −20 °C and can be successfully fractionated even after several days storage. Nuclei frozen during any point in the isolation and/or in storage cannot be subsequently fractionated.

Nuclei were then washed, swollen, and fractionated into condensed (heterochromatin) and extended (euchromatin) regions by the technique of Yasmineh and Yunis (1969, 1970) as previously described (Hardy and Bryan, 1975). Portions of each fraction were stored in 0.001 M Tris (pH 7.4)–50% glycerol at −70 °C and thawed for later physical-chemical characterization. Aliquots for binding studies were directly suspended in appropriate protease inhibitor salt solutions and used immediately.

Characterization of Nuclear Fractions. Thermal Denaturation. Thermal denaturation studies were performed on a Gilford Instruments spectrophotometer. Optical densities and solution temperature (Thermistor) were digitized with a Systron Donnor (7005) digital voltmeter and punched on paper tape with the assistance of a Data Graphics controller (Datos 305). All calculations were sequential 13-point least-squares cubic fits to hyperchromicity, and algebraic computations of the thermal derivatives were performed on a CDC 73 computer. The computer plots (Figures 1 and 2) and calculations have been fully described (Ansevin et al., 1976).

Thermal denaturation of chromatin samples for the assessment of histone–DNA association was performed in 3.6 M urea, 5 mM sodium cacodylate, 1.5×10^{-4} M EDTA, pH 7. Characterization of this analysis can be found (Ansevin and Brown, 1971). Light-scattering corrections throughout these analyses were negligible.

Denaturation analysis of DNA free of protein interactions from chromatin fractions was done at higher resolution using denaturation techniques according to Ansevin (Ansevin et al., 1976) and Vizard and Ansevin (Vizard and Ansevin, submitted). In the reported experiments, the solvent was 0.023 M NaCl, 0.2% sodium dodecyl sulfate, 1.0 mM Tris-HCl, pH 8.0, and 0.1 mM EDTA (30 mM Na^+

[1] Abbreviations: SSC, standard saline citrate (0.14 M NaCl, 0.014 M sodium citrate adjusted to pH 7.0); PMSF, phenylmethanesulfonyl fluoride; Me_2SO, dimethyl sulfoxide; TMKC buffer, 0.05 M Tris, 5.0 mM $MgCl_2$, 3.0 mM KCl, 0.2 mM $CaCl_2$, pH 7.4; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane.

final concentration). Chromatin was homogenized directly into an appropriate concentrate of this solution, cleared of dust by low-speed centrifugation, and degassed at room temperature under vacuum. The solution was then heated at a constant rate of 0.4 °C/min. The use of NaCl–sodium dodecyl sulfate solvent eliminates the need for extensive DNA purification prior to denaturation analysis. Nuclei, chromatin, or highly purified DNA may be used directly with this procedure with no detectable differences in the resulting derivative profiles. Additionally, DNA concentration can be accurately assessed optically for chromatin or nuclei in this solvent as long as the RNA content of the sample is sufficiently low.

Protein–DNA Analysis. Aliquots of Tris-glycerol frozen chromatin fractions were diluted into 1.0% sodium dodecyl sulfate, 8.0 M urea, and 0.05 M Tris, pH 8.0, and absorbance was measured at 260 nm. DNA content was calculated assuming 50 μg of DNA = 1.0 OD at 260 nm. Subsequently, 2.0–4.0-ml aliquots of each fraction were thawed, diluted with 0.5 M HCl to a final concentration of 0.25 M HCl, and vortexed at 4 °C for 20 min. The acid extracted chromatin was spun at 5000 rpm for 10 min at 4 °C and the precipitate (DNA–nonhistone protein complex) solubilized in 1% sodium dodecyl sulfate at 60 °C. The supernatant (histone proteins) was made to 20% Cl_3CCOOH and cooled on ice for 30 min. After spinning at 5000 rpm for 15 min at 4 °C, the pellet was dried and solubilized in 1% sodium dodecyl sulfate. Protein content was measured by the method of Lowry as modified (Schacterle and Pollack, 1973). Protein:DNA ratios were then calculated as milligrams of protein per milligram of DNA in each chromatin fraction.

DNA Isolation and Purification. DNA used in binding studies was purified by the method of Kirby as modified by Elgin and Hood (Elgin and Hood, 1973). Purified DNA was stored at −20 °C in appropriate solutions prior to metal binding analysis.

Quantitation of Metals. Copper was analyzed by flame atomic absorption (Hardy and Bryan, 1975) and mercury, by flameless atomic absorption (Bryan et al., 1974a). A Varian Techtron Type AA5 atomic absorption spectrometer was used with a 51-RU spectrophotometer grating monochromator, a Techtron Dl-30 digital indicator, and a Techtron DC-31 digital corrector [when a recorder was used (Sargent, Model 160), the scale selector was set at 10 mV and the chart speed was 0.5 in./min]. In some experiments, a Perkin-Elmer (Model 360) atomic absorption spectrophotometer with recorder (Houston Instrument Omniscribe) was used.

The copper light source was a hollow cathode tube (Perkin-Elmer Cu 20124) specific for copper with a main resonance line at wavelength 324.7 nm, lamp current 15 mA, and slit width of 0.7 nm. Using air–acetylene flame atomic absorption, levels of copper down to 0.04 μg/ml can be detected; standards ranging from 0.05 to 10.0 μg/ml were prepared from commercially obtained stock solutions (Fisher Scientific).

The mercury light source was either a Jarrel-Ash Type 45493 hollow cathode tube specific for mercury with a main resonance line at wavelength 253.7 nm with a slit width of 100 μm and noise suppression set at 3 or 4, or a Perkin-Elmer type hollow cathode (4104) specific for mercury at 253.6 nm with a lamp current of 10 mA and a slit width of 0.7 nm. Samples were digested prior to mercury analysis by refluxing for 2 h in a concentrated nitric acid–sulfuric acid (3:1) mixture, cooled, diluted to either 25 or 50 ml, and stored in plastic containers (Nalgene) for less than 48 h before mercury determinations were made.

Mercury standards were prepared from a 1000 ppm stock and diluted with 1 N hydrochloric acid (Jarrell-Ash, 1970; Lindsfedt, 1970). Standards were prepared immediately prior to use and a standard curve was plotted for each digestion batch. Immediately prior to determinations of mercury, the reducing agent, 10% (w/v) $SnCl_2$ in concentrated hydrochloric acid, was prepared fresh.

Binding Studies. Equilibrium Dialysis. Freshly isolated heterochromatin and euchromatin or DNA purified from these respective fractions was suspended in either saline (0.9% NaCl)–1% Me_2SO–0.5 mM PMSF or 1:100 saline–1% Me_2SO–0.5 mM PMSF to a DNA concentration of 100 μg/ml. One-milliliter aliquots of each were placed in 0.25-in. diameter visking dialysis casings (exclusion 6000 daltons) previously equilibrated with appropriate solvent and dialyzed up to 8 h against 0.1 mM (6.3 ppm) $CuCl_2$, pH 6.0, or 2.5×10^{-3} mM (0.5 ppm) $HgCl_2$, pH 7.0, in appropriate NaCl–Me_2SO–PMSF solutions; samples were stirred constantly at 25 °C, pH 7.0, and at specified intervals aliquots removed and assayed for DNA, copper, or mercury content.

Gel Filtration. A Sephadex G-25 column (10 mm × 30.0 cm) was equilibrated at 25 ± 2 °C by washing with 200 ml of appropriate metal salt solution as described previously (Bryan et al., 1974b). A sample (freshly isolated heterochromatin and euchromatin or DNA purified from either) was suspended in 1:100 saline–1% Me_2SO–0.5 mM PMSF, pH 6.0 (for copper binding), or 1:100 SSC–1% Me_2SO–0.5 mM PMSF, pH 7.0 (for mercury binding), to a DNA concentration of 100 μg/ml. Samples (1.0 ml) were loaded onto Sephadex G-25 (coarse grade) having a flow rate of 0.5 ml/min, and 0.5-ml fractions were collected and assayed for DNA and metal content.

Results

Properties of Chromatin Fraction. A chemical and physical characterization of the chromatin fractions used in the in vitro binding experiments is given in Table I. A number of these properties cannot be used to distinguish the two nuclear fractions. For example: (1) endogenous levels of copper in both condensed and noncondensed regions of control nuclei are too variable to allow conclusions regarding partitioning of the metal (Hardy and Bryan, 1975); (2) total protein:DNA, histone:DNA, and nonhistone:DNA ratios appear to be similar in the corresponding nuclear fractions; (3) the ultraviolet analyses are compatible with native total chromatin and native DNA; (4) thermal denaturation analyses of total chromatin, total heterochromatin, and total euchromatin show only minor differences (discussed below). However, we present two properties which clearly define the chromatin fractions as unique species: (1) unique partitioning of endogenous mercury into the noncondensed region of the nucleus (Bryan et al., 1974b); (2) localization of mouse satellite DNA primarily in condensed chromatin as revealed by high resolution thermal denaturation analyses of DNA from each fraction.

Thermal Denaturation. The mass fraction of DNA undergoing a helix-to-coil transition as a function of temperature can be used to assess protein–DNA association in urea solvent as described previously (Ansevin and Brown, 1971). Further, the results in sodium dodecyl sulfate solvent can be considered as an assessment of the mass fraction of

Table I: Chemical and Physical Properties of Purified Nuclei and Chromatin Fractions Used in the in Vitro Binding Experiments.

Sample	Endogenous Metal Content[b,c] Mercury[i]	Copper[j]	Protein/ DNA[a]	Uv Absorbance 280/260	Thermal Denaturation Derivative Analysis (°C)[e] Chromatin Max$_1$	Max$_2$	Max$_3$	Max$_4$	Chromatin-DNA Max$_1$	Max$_2$	Max$_3$	Max$_4$
Purified nuclei[d]	0.23 ± 0.05	0.59 ± 0.29										
Total chromatin	0.05[g]	0.15 ± 0.06	*f*	0.59		75	88					
Chromatin-DNA	*f*	*f*		0.50					63	71	73	
Heterochromatin												
Total	0.02 ± 0.01	0.12 ± 0.06	2.72 ± 0.36	0.59		75	88					
Histone fractions			1.69 ± 0.24									
Nonhistone fractions			0.57 ± 0.42									
Heterochromatin-DNA				0.49					63	71	Sh[h]	
Euchromatin												
Total	0.22 ± 0.04	0.20 ± 0.15	2.31 ± 0.12	0.58	Sh[h]	75	88					
Histone fraction			1.36 ± 0.12									
Nonhistone fractions			0.80 ± 0.56									
Euchromatin-DNA				0.49					Sh[h]	71	72	73

[a] No significant differences in protein/DNA (μg of protein/mg of DNA) between corresponding heterochromatin and euchromatin samples (t test, $p < 0.01$). [b] Values represent mean of three experiments ± average deviation from mean. [c] Triton X-100 included in isolation of nuclei. [d] When Triton X-100 was not included in isolation of nuclei: mercury = 0.91 μg of Hg/mg of DNA ± 0.19 and copper = 0.82 μg of Cu/mg of DNA ± 0.39. [e] Max refers to maximum inflection in thermal denaturation profile based on first derivatives. [f] Analysis not made. [g] Gel filtered (see Bryan et al., 1974a; Table I). [h] Shoulder; not resolved into local maximum. [i] In units of μg of Hg/mg of DNA. [j] In units of μg of Cu/mg of DNA.

Table II: Mercury Binding Properties of Nuclei, Chromatin Fractions, and DNA.[a]

A. In Vivo.

	Time Treated[b] with $HgCl_2$ (months) 1	2	3
	$\bar{v}$	$\bar{v}$	$\bar{v}$
Nuclei	*f*	2.66 ± 0.74	*f*
Heterochromatin	0.14 ± 0.09		0.09 ± 0.14
Euchromatin	3.31 ± 0.89	*f*	1.86 ± 0.63

B. In Vitro (pH ~7.0).

	Gel Filtration[c] (Sephadex G-25) Sample	1:100 SSC	SSC[d]		Equilibrium Dialysis[d] 1:100 SSC	SSC
		$\bar{v}$	$\bar{v}$		$\bar{v}$	$\bar{v}$
Free Hg^{2+}	Het	0.58 ± 0.13	*e*	Free Hg^{2+}	0.40 ± 0.14	0.40 ± 0.27
Concn	Euch	4.71 ± 1.04	*e*	Concn 2.5×10^{-6}	3.10 ± 0.15	2.10 ± 0.48
2.5×10^{-5} M	Het-DNA	63.00 ± 7.48	*f*	M (0.5 ppm)	1.40 ± 0.11	*f*
(5.0 ppm)	Euch-DNA	59.40 ± 2.39	*f*		1.60 ± 0.07	
	Calf-thymus DNA[g]	*f*	*f*		*f*	3.65 ± 0.24

[a] Bound mercury $\bar{v}$ = μg of Hg^{+2}/mg of DNA; values represent the mean of two to three experiments ± average deviation from the mean. [b] Animals given 1 mM $HgCl_2$ in drinking water. [c] One-half milliliter of sample containing approximately 100 μg of DNA (unless specified) was added to a Sephadex G-25 column (19 × 1 cm) equilibrated with appropriate mercury and salt solutions at room temperature. [d] One hundred micrograms of chromatin-DNA samples were dialyzed as described in text. [e] Chromatin samples under these conditions would not pass across Sephadex and tended to aggregate and adhere to glass surfaces. [f] Analysis not made. [g] Free Hg^{2+} was 3.1×10^{-5} M.

DNA sequences as described (Vizard and Ansevin, submitted).

The denaturation profiles (Figure 1) show total chromatin, euchromatin, and heterochromatin to be quite similar as would be expected since only very minor differences with respect to histone-DNA associations of euchromatin and heterochromatin have been noted [Frenster (1969); Warneck et al. (1973)]. There is a shoulder near 60 °C in euchromatin, absent in heterochromatin, which likely represents melting of uncomplexed DNA or regions bound by nonhistone protein (Ansevin et al., 1971; Shih and Lake, 1972). However, a substantial difference in the DNA sequences between euchromatin DNA and heterochromatin DNA is clearly reflected in the derivative profiles of these purified DNAs shown in Figure 2. The sequence with a transition at 71 °C which is readily detectable in total chro-

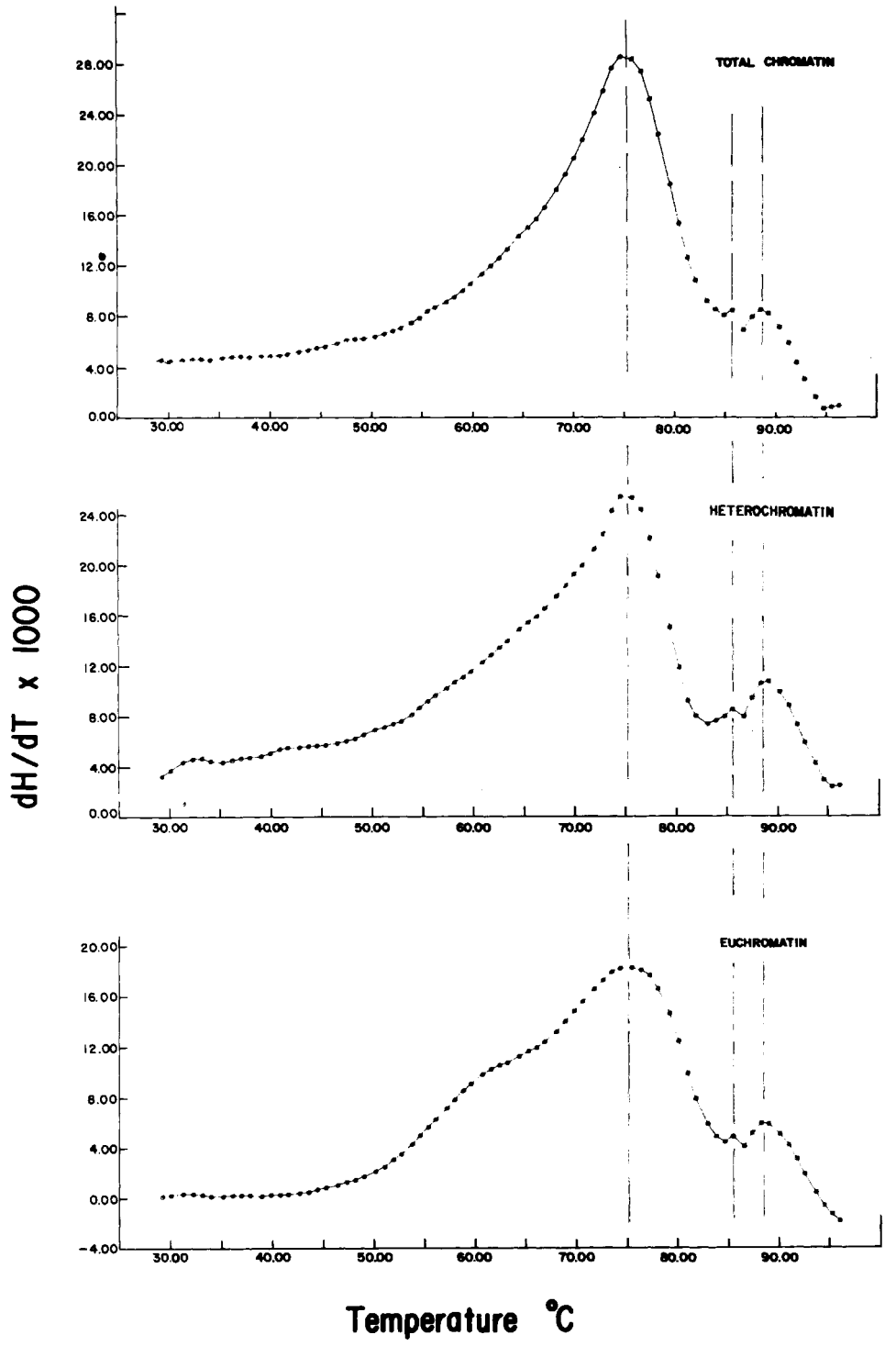

FIGURE 1: Thermal denaturation profiles of total chromatin, heterochromatin, and euchromatin.

matin DNA, and is highly enriched in heterochromatin DNA, corresponds to mouse satellite DNA, well characterized by other techniques such as buoyant density (Yasmineh and Yunis, 1969) and in situ hybridization (Pardue and Gall, 1970). In fact, mouse satellite DNA does undergo denaturation at 71 °C in this solvent (unpublished results). This highly repeated sequence has been used as an important marker of constitutive heterochromatin (Yasmineh and Yunis, 1970).

Mercury Binding Properties in Vivo. Endogenous levels of mercury given in Tables I and II are lower than previously reported for both control and metal-treated animals (Bryan et al., 1974b) in which nuclei had not been isolated in the presence of Triton X-100. That cytoplasmic contaminants bind mercury atoms which are carried in loose association into the chromatin fractions was shown in an earlier paper (Bryan et al., 1974a) where the mercury content ratio of stock to gel-filtered was about 10:1. Nuclei isolated in the absence of a detergent have three to six times more mercury than detergent-treated nuclei (Table I, footnote *d*). We believe that levels of mercury reported in this paper are more representative of endogenous nuclear concentrations. The isolation procedure is otherwise essentially the same as used in previous studies. The metal is now detectable in

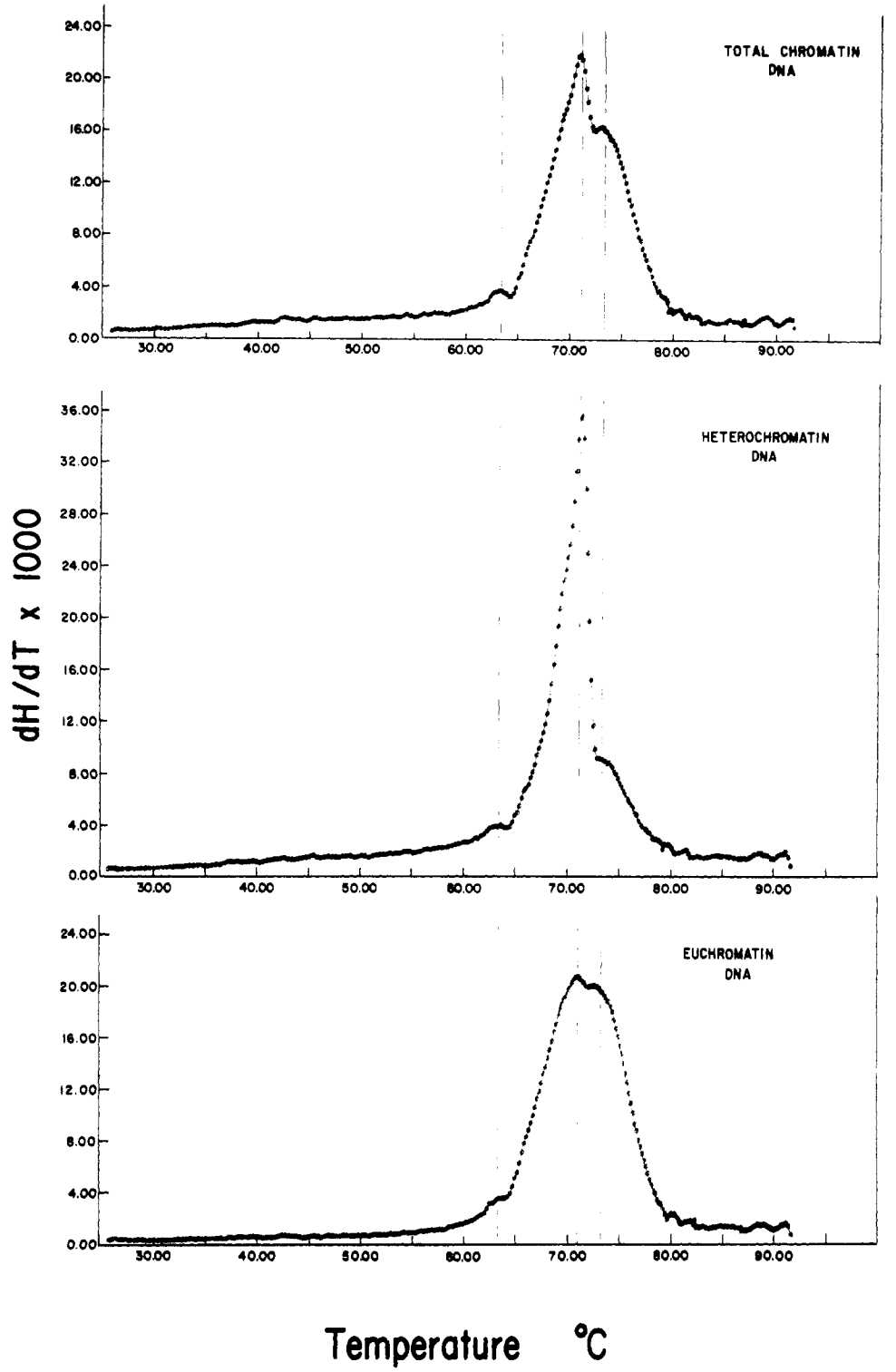

FIGURE 2: Thermal denaturation profiles of total chromatin DNA, heterochromatin DNA, and euchromatin DNA.

trace quantities (0.02 μg of Hg/mg of DNA) in unchallenged animals (Table I) and there is a six- to nine fold accumulation in heterochromatin (relative to control) in challenged animals (Table II). Yet in good agreement with previous findings, nuclear mercury is concentrated in euchromatin as indicated by the euchromatin:heterochromatin ratio which is 10:1 in material from untreated animals (Table I) and about 20:1 in metal challenged animals (Tables II and IV).

Mercury Binding Properties in Vitro. Binding data from both equilibrium dialysis and gel filtration at low ionic strength (1:100 SSC) and from equilibrium dialysis at physiologic ionic strength (SSC) are given in Table IIB, Figure 3, and Figure 4. (Gel filtration could not be used to measure chromatin metal binding under physiologic salt concentrations because of the aggregating properties of the nuclear material, particularly heterochromatin, at this ionic strength.) Euchromatin bound four to ten times more mercury than heterochromatin when measured either in high (SSC) or in dilute (1:100 SSC) salt solutions (Table II-B). Furthermore, the level of mercury bound to euchromatin in vitro is comparable to in vivo bound mercury after 1 month

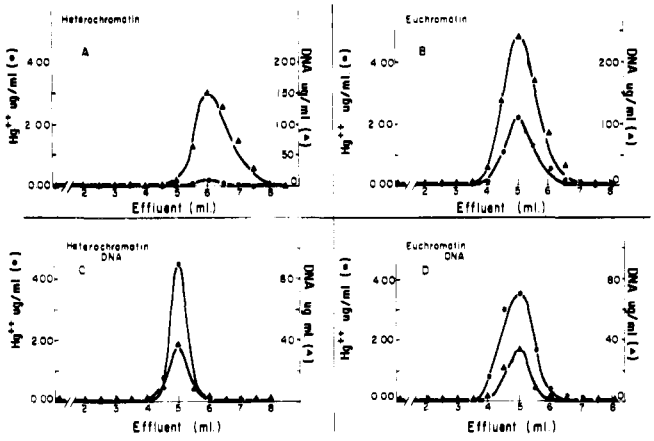

FIGURE 3: Representative mercury binding elution profiles of chromatin fractions. (A) Heterochromatin; (B) euchromatin and DNA isolated from the respective fractions; (C) heterochromatin–DNA; and (D) euchromatin–DNA. One-milliliter samples were applied to Sephadex G-25 (coarse) equilibrated in 2.5×10^{-5} M $HgCl_2$ in SSC/100, pH 7.0, and eluted with the equilibrating buffer.

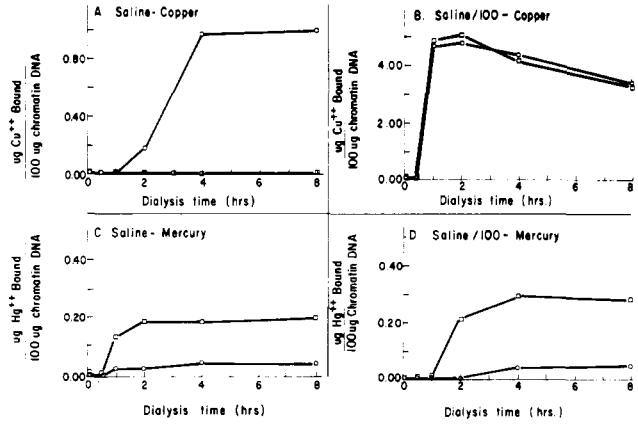

FIGURE 4: Equilibrium dialysis profiles for heterochromatin (○) and euchromatin (□) binding with copper or mercury. (A) Copper (0.1 mM) in saline, pH 6.0; (B) 0.1 mM copper in 1:100 saline, pH 6.0; (C) 2.5×10^{-3} mM mercury in saline, pH 7.0; (D) 2.5×10^{-3} mM mercury in 1:100 saline pH 7.0.

of challenge with dietary metal. The nuclear fractions bound essentially the same amount of metal when equilibrated with either 0.5 ppm (5.0 μg/mg of DNA) or 5 ppm (50 μg/mg of DNA) suggesting a saturation of binding sites (Table II-B).

On the other hand, when heterochromatin and euchromatin were stripped of proteins and the DNA purified from each gel-filtered in the presence of free mercury, specificity of binding previously observed was abolished (Table II-B and Figure 3, C and D). Under these conditions DNA from both fractions bound the same amount of mercury. Furthermore, when DNA from chromatin fractions was dialyzed against free mercury at 0.5 ppm, which approximates concentrations seen in euchromatic portions of challenged animals (Table II-B), less binding to purified DNA was observed than to the total DNA–protein–euchromatin complex at the same mercury level (1.60 μg of Hg/mg of DNA vs. 3.10 μg of Hg/mg of DNA, respectively). Conversely, at higher free mercury levels (5.0 ppm), DNA purified from chromatin fraction bound greater than ten times more mercury per mg of DNA than did the euchromatin complex under identical conditions (Table II, B). Thus, in either concentration of free nuclear mercury, the extent of binding is restricted by protein species unique to the particular chromatin fraction. Additionally, specificity of binding is independent of ionic strength and, hence, relative state of chromatin condensation. Consequently heterochromatin consistently bound less mercury than euchromatin even when heterochromatin was fully dispersed (in 1:100 SSC) (Figure 3, Table II).

Copper Binding Properties in Vivo. In accord with previous observations (Hardy and Bryan, 1975) there appears to be an enrichment of copper in heterochromatin (relative to euchromatin) when animals are challenged with dietary copper for 1 month; after 2 months of exposure there is an increased but similar partitioning of metal into nuclear compartments (Table IIIA). Euchromatin bound copper remains relatively constant (0.25 ± 0.10 and 0.41 ± 0.16) displaying no apparent enrichment in response to challenge while levels of the metal in heterochromatin seem to be subject to wider variations (1.42 ± 0.96 and 4.94 ± 2.96). Both challenged and unchallenged animals show greater variations in nuclear copper than in nuclear mercury [Tables IA and IIA].

Copper Binding Properties in Vitro. In diluted salt (1:100 saline), copper readily binds to both heterochromatin and euchromatin (Figure 4B, Table IIIB) exhibiting little or no relative preference for the nuclear fractions. Utilizing equilibrium dialysis under relatively physiological conditions (saline), a preferential binding pattern mimics that observed in vivo; i.e., heterochromatin binds copper to a measurable extent, whereas euchromatin displays no appreciable binding (Figure 4B).

The binding of Cu(II) to purified DNA is highly ionic-strength dependent (Figure 5): strong interactions occur only in dilute salt solutions, and there is essentially no copper–DNA binding at physiological ionic strength. These results are in accord with previous observations (Bryan and Frieden, 1967) and suggest that copper binding sites in the nucleus are probably protein in nature but one cannot rule out DNA-mediated protein interactions. It is possible that copper could bind to DNA in a DNA–protein complex under conditions which would produce no apparent binding to DNA alone. It is likely that at low ionic strengths chromatin decondensation occurs resulting in more direct DNA–copper binding. This is supported by data from gel filtration binding of copper to DNA purified from either of the chromatin fractions (Figure 4B) in which binding specificity is essentially lost. There is slightly more copper bound to each mg of DNA under these conditions than to the chromatin complex (Table III B), suggesting that even at low ionic strengths a considerable portion of the chromatin bound DNA is unavailable for binding to copper.

Discussion

The fractionation of chromatin into active and inactive portions can be achieved by a number of techniques (Duerksen and McCarthy, 1971; Frenster et al., 1963; Yasmineh and Yunis, 1970). Methods based on differential centrifugation of sonicated nuclei are used in the present study to separate chromatin into condensed (inactive) and noncondensed (active) fractions of heterochromatin and euchromatin, respectively. DNAs isolated from these fractions differ in a number of characteristics. For example, the mouse genome contains an easily distinguishable satellite DNA highly redundant in base sequences (Waring and

Table III: Copper Binding Properties of Nuclei, Chromatin Fractions, and DNA.[a]

A. In Vivo.

	Time Treated[b] with $CuCl_2$ (Months)		
	1	2	3
	$\bar{v}$	1.95 ± 1.15	$\bar{v}$
Nuclei	1.60 ± 1.01		*g*
Heterochromatin	1.42 ± 0.96	4.94 ± 2.96	
Euchromatin	0.25 ± 0.10	0.41 ± 0.16	

B. In Vitro.

		Gel Filtration[c] (Sephadex G-25)				
				Equilibrium Dialysis[d]		
Free Cu^{2+} Concn	Sample	1:100 Saline	Saline	Free Cu^{2+} Concn	1:100 Saline	Saline
		$\bar{v}$			$\bar{v}$	$\bar{v}$
1.0×10^{-5} M	Het	2.34 ± 0.30	*f*	1.0×10^{-4} M	44.00 ± 3.60	10.00 ± 0.89
(0.63 ppm)	Euch	3.57 ± 0.10	*f*	(6.3 ppm)	42.00 ± 4.10	*e*
1.0×10^{-4} M	Het-DNA	70.50 ± 7.30	*e*			
(6.3 ppm)	Euch-DNA	57.25 ± 1.75	*e*			
1.0×10^{-4} M	Calf-thymus					
(6.3 ppm)	DNA	40.30 ± 3.75	*e*			

[a] Bound copper $\bar{v}$ = μg of Cu^{2+}/mg of DNA; values represent the mean of two to three experiments ± average deviations from the mean. [b] Animals given 10 mM $CuCl_2$ in drinking water. [c] One-half milliliter of sample containing approximately 100 μg of DNA was added to Sephadex G-25 (coarse) column (19 × 1 cm) equilibrated with appropriate copper and salt solution. [d] One hundred micrograms of chromatin-DNA samples were dialyzed as described in text. [e] No detectable binding. [f] Chromatin samples under these conditions would not pass across Sephadex and tended to aggregate and adhere to glass surfaces. [g] Analysis not made.

Table IV: Mercury Enrichment in Chromatin Fractions In Vivo.[a]

Expt	Animal No.	Treatment Time (Weeks)	Mercury μg/mg of DNA Exptl/Control		Euchromatin Enrichment Ratio[c]	
			Het	Euch	Het	Euch
1	20	4	*d*	12.5		
2	10	4	7.0	15.0	1.0	23.6
3	20	7	*d*	15.1		
4	10	15	4.5	8.5	1.0	20.6
5	13	20[b]	*d*	10.1		

[a] Animals treated with 1 mM $HgCl_2$ in drinking water. [b] Animals treated with 1 mM $HgCl_2$ in drinking water from birth. [c] Enrichment ratio: mercury content of sample:mercury content of heterochromatin within an experiment. [d] Mercury was not detected in heterochromatin (less than 0.5×10^{-3}).

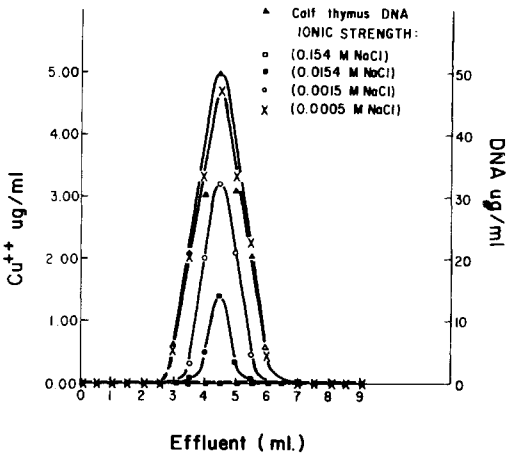

FIGURE 5: Ionic strength dependence of DNA-Cu binding at pH 7.0. Sephadex G-25 was equilibrated with 0.1 mM $CuCl_2$, pH 7.0, ionic strength as indicated.

Britten, 1966) localized in the heterochromatin portion of the nucleus (Yunis and Yasmineh, 1970). Our derivative thermal denaturation profile analyses have revealed distinct mouse satellite DNA sequences in heterochromatin (as compared with total chromatin and euchromatin, Figure 2) and have clearly identified the chromatin fractions used in the binding experiments. Mercury is also a useful marker in the identification of euchromatin and heterochromatin. Under either control or metal-challenged conditions, the localization of mercury in euchromatin is pronounced relative to heterochromatin, that is 10:1 and 20:1, respectively. That these nuclear constituents display unique binding affinities toward both copper and mercury is shown in this paper and in earlier reports (Bryan et al., 1974b; Hardy and Bryan, 1975). In the present investigation we have measured specificity of binding in vitro to condensed and noncondensed chromatin under conditions which mimic the in vivo state.

The specificity of mercury binding is primarily determined by the association of protein with DNA. When proteins are removed and mercury binding measured for heterochromatin DNA and euchromatin DNA, equivalent binding to the DNA fractions is observed: $\bar{v}$ = 63.0 and 59.4 at 5.0 ppm of free Hg^{2+}, and $\bar{v}$ = 1.40 and 1.60 at 0.5 ppm of free Hg^{2+}. When levels of free mercury are approximately ten times (5.0 ppm of free Hg^{2+}) that found in the mercury-challenged state in vivo, binding of the metal to free DNA is much greater than binding to protein associated DNA. This restriction and specificity of binding is given by nuclear proteins. When levels of free mercury (0.5 ppm) approximate those found in the mercury challenged in vivo nucleus (5 μg/mg of DNA), about one-half as much mercury binds to free euchromatin DNA as binds to the same

DNA when proteins are associated, suggesting specific binding sites, probably protein in nature, within euchromatin. Heterochromatin, on the other hand, binds nearly four times more mercury once proteins are removed. Therefore, heterochromatin regions, while restrictive in nature, probably fail to contain the mercury binding proteins reported to exist in total chromatin (Chanda and Cherian, 1973). While binding of mercury to free DNA is lower at higher salt concentrations (calf thymus data, Table II), it is nevertheless clear that mercury could interact with DNA under physiologic conditions unless otherwise directed through protein associations.

Removal of proteins from the chromatin fractions also results in a loss of metal binding specificity for copper and elicits enhanced binding similar to those observed for copper bindings to heterochromatin and euchromatin at low ionic strength. Therefore, in low salt solutions, copper probably binds predominantly to DNA, whereas at physiological ionic strength direct DNA binding is not favored, but the possibility of a protein-DNA directed binding cannot be discounted. The in vivo copper sites are probably not completely saturated as evidenced by variability in amount of copper bound (from experiment to experiment) and by the observation that heterochromatin binds about three times more copper in vitro (in saline against 6.3 ppm of free copper, $\bar{\nu} = 10.0$ μg of Cu^{2+}/mg of DNA) than was observed in vivo ($\bar{\nu} = 3.7$ μg of Cu^{2+}/mg of DNA).

Thus, in this investigation we have shown that condensed and noncondensed chromatin fractions interact with copper and mercury under relatively physiologic conditions in vitro with binding specificity similar to that observed in in vivo experiments. The binding selectivity in both cases appears to arise from protein or protein-DNA association. Additionally, the state of chromatin condensation as predetermined in vitro by ionic strength is critical to selectivity of binding of one heavy metal, copper, yet of considerably less importance to binding selectively toward mercury. Conceivably, therefore, differences in extent of nuclear chromatin condensation whether due to either tissue differentiation, a normal physiologic response, or to some pathological abnormality, could coincide with alterations in selectivity of metal localization and might be correlated with changes in gene expression. That mercury localizes in euchromatin irrespective of state of chromatin condensation is probably indicative of the presence of a select mercury binding protein, or class of proteins. Indeed this has already been suggested in rat liver total chromatin (Chanda and Cherian, 1973). Tissue and site specific nuclear binding proteins have been recently demonstrated and implicated in the regulation of tissue specificity (Chiu et al., 1975; Wakabayashi et al., 1974). Other recent observations have suggested that these gene regulatory proteins are localized predominantly in extended chromatin (Dr. J. F. Chiu, personal communications).

Several lines of evidence suggest that steroid hormones, the best known effectors of gene regulation, localize in the nucleus by binding to chromatin possibly in the form of a hormone-protein (receptor) complex (Spelsberg, 1974; O'Malley et al., 1972). It is possible that there are other "effectors" of this type which bind to chromatin. A metal such as copper or mercury, which appears to localize in the nucleus by binding at specific sites on chromatin could possibly act as an "effector" in gene regulation. The possibility cannot be overlooked that metal nuclear accumulation is a physiologic response which may even be part of a specific mechanism of gene activation or repression. For example, a nuclear protein kinase which is present only in tumorogenic tissues and which functions only in the presence of low levels of Mn^{2+} has been recently demonstrated (Thomson et al., 1974, 1975). Accordingly, trace levels of copper and mercury shown to be present in control could be related to some unknown co-factor function.

Since metal ions are involved in essentially every phase of genetic information transfer, it is only logical to suspect important structural and/or functional roles for copper and perhaps even mercury in the eukaryotic nucleus. The elucidation of such roles provides challenging problems for the future.

Acknowledgments

The authors acknowledge with thanks the hospitality given by Dr. A. T. Ansevin in making the thermal denaturation studies possible and are grateful to Mr. Harvey J. Fitzpatrick for technical assistance.

References

Ansevin, A. T., and Brown, B. W. (1971), *Biochemistry 10,* 1133.

Ansevin, A. T., Hnilica, L. S., Spelsberg, T. C., and Kehm, S. L. (1971), *Biochemistry 10,* 4793.

Ansevin, A. T., Vizard, D. L., Brown, B. W., and McConathy, J. (1976), *Biopolymers* (in press).

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

Blobel, G., and Potter, V. A. (1966), *Science 154,* 1662.

Bryan, S. E., and Frieden, E. (1967), *Biochemistry 6,* 2728.

Bryan, S. E., Guy, A. L., and Hardy, K. J. (1974a), *Biochemistry 13,* 313.

Bryan, S. E., Lambert, C., Hardy, K. J., and Simons, S. (1974b), *Science 186,* 832.

Chanda, S. K., and Cherian, G. (1973), *Biochem. Biophys. Res. Commun. 50,* 1013.

Chiu, J. F., Hunt, M., and Hnilica, L. S. (1975), *Cancer Res. 35,* 913.

Clark, R. J., and Felsenfeld, G. (1974), *Biochemistry 13,* 3622.

Duerksen, J. D., and McCarthy, B. J. (1971), *Biochemistry 10,* 1471.

Elgin, S. C. R., and Hood, L. E. (1973), *Biochemistry 12,* 4984.

Frenster, J. H. (1969), Handbook of Molecular Cytology, Lima-de-Faria, A., Ed., London, North-Holland Publishing Co., p 251.

Frenster, J. H., Allfrey, V. G., and Mirsky, A. E. (1963), *Proc. Natl. Acad. Sci. U.S.A. 50,* 1026.

Gilmour, R. S. (1974), Acidic Proteins of the Nucleus, New York, N.Y., Academic Press, p 297.

Hardy, K. J., and Bryan, S. E. (1975), *Toxicol. Appl. Pharmacol. 33,* 62.

Jarrell-Ash (1970), Atomic Absorption Analytical Applications, No. Hg-1, Fisher, Waltham, Mass., Jarrell-Ash.

Lindsfedt, G. (1970), *Analyst 95,* 264.

Nandi, U. S., Wang, J. C., and Davidson, N. (1965), *Biochemistry 4,* 1687.

O'Malley, B. W., Spelsberg, T. C., Schrader, W. T., Chytyl, F., and Steggles, A. W. (1972), *Nature (London) 235,* 141.

Pardue, M. L., and Gall, J. G. (1970), *Science 168,* 1356.

Schacterle, G. R., and Pollack, R. L. (1973), *Anal. Bio-*

chem. 51, 654.
Shih, T. Y., and Lake, R. S. (1972), *Biochemistry 11,* 4811.
Spelsberg, T. C. (1974), Acidic Proteins of the Nucleus, New York, N.Y., Academic Press, p 262.
Spelsberg, T. C., Wilhelm, J. A., and Hnilica, L. S. (1972), *Sub-Cell. Biochem. 1,* 107.
Stein, G. S., and Farber, J. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 2918.
Stein, G. S., Spelsberg, T. C., and Kleinsmith, L. J. (1974), *Science 183,* 817.
Thomson, J. A., Chiu, J. F., and Hnilica, L. S. (1974), *J. Cell Biol. 63,* 348a.
Thomson, J. A., Chiu, J. F., and Hnilica, L. S. (1975), *Proc. Am. Assoc. Cancer Res. 16,* 158.
Vizard, D. L., and Ansevin, A. T., submitted for publication.
Wagner, T. E., and Vandergrift, V. (1972), *Biochemistry 11,* 1431.
Wakabayashi, K., Wang, S., and Hnilica, L. S. (1974), *Biochemistry 13,* 1027.
Waring, M., and Britten, R. J. (1966), *Science 154,* 791.
Warneck, P., Krus, K., and Harbers, E. (1973), *Biochim. Biophys. Acta 331,* 295.
Wilhelm, J. A., Spelsberg, T. C., and Hnilica, L. S. (1971), *Sub-Cell. Biochem. 1,* 39.
Yasmineh, W. G., and Yunis, J. J. (1969), *Biochem. Biophys. Res. Commun. 35,* 779.
Yasmineh, W. G., and Yunis, J. J. (1970), *Exp. Cell Res. 59,* 69.
Yunis, J. J., and Yasmineh, W. G. (1970), *Science 168,* 263.
Zimmer, C. H., Luck, G., Fritzsche, H., and Triebel, H. (1971), *Biopolymers 10,* 441.

Interactions between Model Proteins and Deoxyribonucleic Acids

Margaret F. Pinkston, Andrew H. Ritter, and Hsueh Jei Li*

ABSTRACT: Interactions between DNA and model proteins, poly(L-Lys^{m}L-Ala^n), where $m + n = 100\%$, have been investigated using thermal denaturation and circular dichroism (CD). All complexes of DNA with these proteins precipitate in a small range of input ratios, protein to DNA, with the midpoints of all precipitation curves close to a 1:1 ratio of lysine to phosphate. The melting temperature of model protein-bound DNA regions decreases slightly as the alanine content of the model protein is increased, which can be explained as a result of insufficient charge neutralization of phosphates by lysine residues in the model proteins. In the free state, these model proteins possess varying amounts of α helix, random coil, or a mixture of these two, depending upon the relative lysine/alanine content. When bound to DNA, the CD of the complex shows a substantial increase in α-helical structure for those model proteins with 40–60% alanine, while there is no significant change in α-helical structure when the percent alanine is either substantially higher or lower (i.e., 81 or 19% alanine). Only those complexes formed with model proteins having 40–60% alanine undergo a drastic transition from a B-type CD to an A-type in the presence of intermediate ionic strength (0.2 M NaCl, for example). Poly(Lys^{19}Ala^{81})·DNA complexes show a slight transition toward A-type CD at 0.4 M NaCl or higher. Apparently other factors, in addition to alanine and α-helical content, must be responsible for this B $\rightarrow$ A transition. At the other extreme of lysine/alanine ratio, with high lysine content, poly(Lys^{81}Ala^{19}) or polylysine, the presence of NaCl produces a B $\rightarrow \Psi$ transition. The possible significance of these differences in response to the binding of these model proteins is discussed.

Protein-DNA interactions relate closely at the molecular level to both chromosome structure and gene regulation. Interactions between DNA and proteins, such as histones and the lactose repressor, have been studied in many laboratories (Hnilica, 1972; Johnson et al., 1974; Von Hippel and McGhee, 1972). In order to understand these interactions, model proteins, in which such parameters as amino acid composition, sequence, and secondary structure can be controlled, have been investigated (Friedman and Ts'o, 1971; Sponar et al., 1974; Santella and Li, 1974; Pinkston and Li, 1974; Mandel and Fasman, 1974; Stokrova et al., 1975; Santella and Li, 1975).

Previously we reported the results of thermal denaturation and circular dichroism (CD) studies on poly(Lys^{40}Ala^{60})-DNA complexes (Pinkston and Li, 1974). Poly(Lys^{40}Ala^{60}) contains some degree of α-helical structure in the free state. When it is complexed with DNA, its α-helical content is markedly increased while the DNA conformation moves from B slightly toward C form. Change in the ionic environment of the complex by the addition of NaCl induces a further structural transition of the DNA from partial C form toward A conformation. In order to probe more extensively the effects of complex formation on the secondary structures of both model proteins and DNA, as well as the structural effects due to changes in ionic conditions, interactions between DNA and a series of related model proteins, poly(Lys^{m}Ala^n), where $m + n = 100\%$, were studied by means of thermal denaturation and CD. Variation in the lysine and alanine content of the

† From the Department of Chemistry, Brooklyn College of the City University of New York, Brooklyn, New York 11210, and Division of Cell and Molecular Biology, State University of New York at Buffalo, Buffalo, New York 14214. *Received October 17, 1975.* Supported by Grant GM21481 from U.S. Public Health Service.
* Address correspondence to this author at the State University of New York at Buffalo.