Circular Dichroic and Sedimentation Studies of Phosphorylated H1 from Chinese Hamster Cells†

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ABSTRACT: This paper presents the first study of the conformational changes of histone H1 phosphorylated in vivo. Phosphorylated histone H1 ($H1_P$) was isolated from Chinese hamster (line CHO) cell cultures synchronously enriched in metaphase cells, and unphosphorylated H1 ($H1_0$) was isolated from cells arrested in G_1 by isoleucine deprivation. Circular dichroic measurements of CHO $H1_P$, CHO $H1_0$, and calf thymus H1 indicate that (a) the cell-cycle-dependent phosphorylations of $H1_P$ do not alter the sensitivity of CHO H1 to undergo salt-induced folding in solution; (b) the net secondary structure of folded H1 is not affected by H1 phosphorylation; (c) the presence of divalent cations (which might bind to the phosphates of $H1_P$) does not alter H1 folding or the folded conformation of $H1_P$; and (d) the net secondary structure of folded CHO H1 is the same as that of calf thymus H1 and involves about 15% of the H1 residues. Sedimentation measurements of phosphorylated or unphosphorylated H1 provide no evidence of H1:H1 interactions in solution. Finally, H1:DNA complexes of CHO $H1_0$ or $H1_P$ and PM2 DNA [80% superhelical (type I); 20% nicked, closed, circular (type II)] were prepared by direct mixing. Sedimentation boundary measurements as a function of sodium chloride concentration show that both $H1_0$ and $H1_P$ induce formation of a 140 ± 20S component and of large heterogeneous aggregates (≥1500 S). While the salt concentrations required to induce these sedimenting species are similar for both $H1_0$ and $H1_P$, the circular dichroic spectra of $H1_0$:DNA and $H1_P$:DNA in the aggregated complexes (120 mM NaCl) are different from one another. These studies indicate that the cell-cycle-dependent phosphorylations of histone H1 have little effect upon H1 conformation, H1:H1 interactions, or ability of H1 to induce aggregation of DNA in H1:DNA complexes as a function of sodium chloride concentration. Nevertheless, circular dichroic spectra of aggregated H1:DNA complexes indicate that H1 phosphorylation does alter the interaction of H1 with DNA.

It has been suggested for several years that histone H1 is involved in chromatin condensation (Bradbury et al., 1973a) and that H1 mitotic phosphorylation may be a necessary condition (Lake, 1973; Gurley et al., 1974) or a trigger mechanism (Bradbury et al., 1973b) for chromosomal condensation during mitosis. Although the roles of H1 phosphorylation are unresolved, recent data indicate that histone H1 is necessary for the organization of nucleosomes into higher orders of chromatin structure. H1 appears to be bound, at least in part, to internucleosomal spacer regions (Shaw et al., 1976; Varshavsky et al., 1976; Whitlock & Simpson, 1976; Noll & Kornberg, 1977). It is required for the folding of oligonucleosomes into solenoids (Finch & Klug, 1976) and for formation of the 200–300-Å native-like chromatin fiber (Renz et al., 1977).

Previous physical studies of histone H1 have employed calf thymus H1, which is unphosphorylated, or calf thymus H1, which has been enzymatically phosphorylated in vitro. Smerdon & Isenberg (1976a) separated unphosphorylated calf thymus H1 into a number of primary-structure subfractions. These subfractions undergo similar conformational changes as a function of salt concentration (Smerdon & Isenberg, 1976a); yet they interact with the nonhistone proteins HMG1 and HMG2 in a specific fashion (Smerdon & Isenberg, 1976b; Yu & Spring, 1977). The research group at Portsmouth, England (Hartman et al., 1977, and references therein), has extensively studied H1 fragments produced by proteolytic cleavage. Their data indicate that, in salt solution, calf thymus H1 can be considered as being composed of three structural regions: "a random coil "nose" consisting of 35–40 residues from the NH_2-terminal end; a globular "head" involving the next approximately 80 residues; and a random "tail" of the remainder of the molecule" (Hartman et al., 1977).

Histone H1 enzymatically phosphorylated in vitro has been used in a number of physical studies (Adler et al., 1972; Glotov et al., 1977; Matthews & Bradbury, 1978). These studies have employed H1 phosphorylated at "serine-37" (Adler et al., 1972; Glotov et al., 1977; Matthews & Bradbury, 1978), at "serine-106" (Adler et al., 1972; Matthews & Bradbury, 1978), and at a number of sites phosphorylated with a "growth-associated" kinase (Matthews & Bradbury, 1978) isolated from Ehrlich ascites cells (Langan, 1976). Phosphorylation at "serine-37" has been observed in vivo at low levels involving only about 1% of the H1 molecules (Langan & Hohmann, 1975; Hohmann, 1978). This phosphorylation is cAMP-dependent and can be induced by hormonal stimulation (Langan & Hohmann, 1975). It does not appear to occur in Chinese hamster (line CHO) cells, however (see below). Phosphorylation at "serine-106" (Langan & Hohmann, 1975) has not been correlated with in vivo phosphorylation events (Matthews & Bradbury, 1978; Hohmann, 1978). While H1 phosphorylations of the "growth-associated" kinase (Langan, 1976) appear to be similar to those that occur in vivo (Hohmann et al., 1976), this has not yet been demonstrated (Matthews & Bradbury, 1978).

Histone H1 undergoes a number of cell-cycle-dependent phosphorylations in proliferating cells (Bradbury et al., 1973b; Gurley et al., 1973a, 1975; Lake, 1973). CHO H1 is phosphorylated during both interphase and mitosis (Gurley et al., 1973a,b; 1975). During interphase, a maximum of 60% of the H1 molecules are phosphorylated at 1–3 serines in the COOH-terminal fragment of H1 as revealed by bisection at tyrosine with *N*-bromosuccinimide (NBS)[1] (Gurley et al., 1975, 1978a; Hohmann et al., 1976). During mitosis, all H1

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[1] Abbreviations used: CD, circular dichroism; CT, calf thymus; EDTA, ethylenediaminetetraacetic acid; NBS, *N*-bromosuccinimide; ^1H NMR, proton magnetic resonance; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; cAMP, cyclic adenosine 3′,5′-monophosphate.

0006-2960/79/0418-0943$01.00/0

molecules are phosphorylated at additional serines and threonines in both the NH_2-terminal and COOH-terminal NBS fragments so that there are 4–6 phosphates per molecule (Hohmann et al., 1976). The highly phosphorylated H1 containing 4–6 phosphates ($H1_M$) occurs only during prophase, metaphase, and anaphase when chromosomes are maximally condensed, and $H1_M$ is dephosphorylated during early telophase when chromosomes begin to unravel (Gurley et al., 1978a). It should be noted that, since phosphorylation is not observed in the NH_2-terminal NBS fragment of H1 during interphase, phosphorylation at serine-37 does not appear to occur in CHO cells (Hohmann et al., 1976).

Temporal correlations of H1 phosphorylations during the cell cycle have led to suggestions that H1 phosphorylations may play a role in cell proliferation (Ord & Stocken, 1968; Stevely & Stocken, 1968; Balhorn et al., 1972; Gurley et al., 1973a, 1975), in DNA replication (Oliver et al., 1972; Ajiro et al., 1975; Gurley et al., 1975), and in chromosomal condensation (Bradbury et al., 1973b; Lake, 1973; Marks et al., 1973; Gurley et al., 1974, 1975, 1978a; Ajiro et al., 1975). A number of mechanisms have been suggested by which H1 phosphorylation might fulfill these roles: (a) H1 phosphorylation may induce changes in H1 conformation (Stevely & Stocken, 1968; Langan & Hohmann, 1975; Hohmann et al., 1976) that could alter the orientation of nucleosomes (Gurley et al., 1978a); (b) phosphorylation may induce H1:H1 interactions (Bradbury et al., 1973a, 1974); and (c) H1 phosphorylation may alter H1 interaction with DNA (Ord & Stocken, 1968; Adler et al., 1972; Dixon et al., 1975). The data of Smerdon & Isenberg (1976b) raise the possibility that H1 phosphorylations may also modulate the interaction of H1 with HMG or other nonhistone chromosomal proteins.

In this report, we compare a number of properties of phosphorylated H1 ($H1_P$) isolated from cultures synchronously enriched in metaphase cells with those of unphosphorylated H1 ($H1_0$) isolated from G_1-arrested cells (Gurley et al., 1975). Circular dichroism (CD) and sedimentation methods have been employed in these investigations. The phosphorylations of $H1_P$ do not affect the sensitivity of H1 folding; nor do they affect the net secondary structure of the folded H1. They do not induce dimerization or aggregation of H1 in solution; nor do they greatly alter the salt concentration required to induce aggregate formation of H1:DNA complexes.

Experimental Procedures

Cell Culture and Cell Synchrony. Suspension cultures of Chinese hamster cells (line CHO) were grown in F-10 medium supplemented with 15% newborn calf serum, streptomycin, and penicillin (Tobey et al., 1966). Cultures were maintained free of *Mycoplasma* contamination, determined by periodic assay, as previously described (Walters et al., 1974). Cell concentrations in the cultures were determined with an electronic particle counter (Tobey et al., 1967).

CHO cells for histone isolation were grown in 4-L suspension cultures. Cells containing unphosphorylated $H1_0$ were obtained by synchronizing cultures in G_1 arrest using the isoleucine deprivation method of Tobey & Ley (1971). Cells containing phosphorylated $H1_P$ were obtained from cultures synchonously enriched in metaphase cells (Gurley et al., 1975). Such cultures were produced by first synchronizing exponentially growing cells in G_1 by maintenance in isoleucine-deficient medium for 36 h. These cells were resuspended in complete F-10 medium containing mM hydroxyurea, which resynchronized the cells near the G_1/S boundary. After 10 h in hydroxyurea, the cells were resuspended in complete F-10 medium. At 4 h following release from the hydroxyurea blockade, Colcemid was added (0.067 μg/mL) so that cells progressing into mitosis became arrested in metaphase. Cultures were harvested 6 h after addition of Colcemid. The mitotic fraction, determined by phase contrast microscopy of fixed cells (Gurley et al., 1978a), was typically 67%. It has been shown (Gurley et al., 1973b) that most of the remaining cells in such cultures are in S and G_2 and, thus, contain phosphorylated H1 of the interphase type (Hohmann et al., 1976). The cultures contain the maximum number of mitotic cells obtainable in a large-volume suspension culture, because CHO cells escape from the blockade in metaphase after 5–6 h in the presence of Colcemid (Stubblefield, 1964; Tobey et al., 1972). While higher synchrony (≥90%) is obtainable by mitotic selection (Tobey et al., 1967), this procedure yields insufficient material for physical studies of histone H1.

Histone Isolation. Histones were prepared from CHO cells by the first method of Johns (1964) as adapted to CHO cells by Gurley & Hardin (1968); however, sodium bisulfite (Bartley & Chalkley, 1970) and mercaptoethanol were added to the appropriate solutions (Gurley et al., 1975). Acetone-precipitated H1 was dissolved in water, lyophilized, and stored in desiccated containers at −16 °C for further purification. Calf thymus H1 was also prepared by the first method of Johns (1964) using sodium bisulfite and mercaptoethanol in the appropriate solutions.

H1 was freed of the nonhistone proteins HMG1 and HMG2 by chromatography on Bio-Rex 70 (Kincade & Cole, 1966) as follows. H1 (7–12 mg) from the Johns procedure (1964) was dissolved in 8.0% guanidine hydrochloride (Schwarz/Mann Ultrapure) in 0.10 M phosphate buffer (pH 7.2) and applied to a Bio-Rex 70 column (2 × 17 cm) equilibrated with the same buffer. After the loaded column was washed with 160 mL of the starting buffer, H1 was eluted with 10.25% guanidine hydrochloride in 0.10 M phosphate buffer. Protein in the effluent was detected by absorbance at 218 nm (Hohmann et al., 1976), and those fractions containing protein were desalted on a 2 × 40 cm column of Sephadex G-25 equilibrated with 1 mM HCl and then lyophilized. At this point, $NaDodSO_4$ gels indicated that the material was 95–96% H1. The salt-free material was applied to a 1.3 × 180 cm column of Sephadex G-100 equilibrated with 1 mM HCl to remove remaining impurities. The first two-thirds of the H1 peak was collected and lyophilized. As a final step, the H1 was passed through a Sephadex G-25 column equilibrated with 1 mM HCl, lyophilized, and stored in desiccated containers at −16 °C. The G-25 column was necessary to remove dust and/or resin particles that caused long wavelength scattering in the absorbance spectrum. Quantities of 3–4 mg of highly purified H1 were obtained with these procedures.

Electrophoresis. Electrophoresis of H1 was performed on 15% sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gels (Laemmli, 1970). H1 (5 μg) in 25 μL of $NaDodSO_4$ sample buffer was applied to the $NaDodSO_4$ gels, which were run for 1 h at 60 V and then for 3.5 h at 120 V. The gels were stained for 3 h with 0.25% Coomassie brilliant blue R-250 (Bio-Rad Laboratories) in 45% methanol–9% acetic acid and destained by diffusion in 5% methanol–10% acetic acid.

H1 was also subjected to electrophoresis on long (0.6 × 25 cm) acetic acid–urea–polyacrylamide gels (Panyim & Chalkley, 1969a) as previously described (Gurley et al., 1978a). CT-H1 was loaded on the gels 4 h prior to loading with CHO H1 sample to establish a mobility reference for the various CHO H1 bands (Gurley et al., 1978a).

Densitometer profiles of the $NaDodSO_4$ and acid–urea gels were measured with a Gilford Model 240 spectrophotometer

equipped with a gel linear transport device. Different H1 bands were resolved and quantified electronically with a Du Pont Model 310 curve resolver (Panyim & Chalkley, 1969b).

Circular Dichroism Measurements. Circular dichroic spectra were measured at 22 °C with a JASCO J-40C spectropolarimeter. The instrument was calibrated with *d*-camphor-10-sulfonic acid (Aldrich) using $\Delta\epsilon = 0.236$ cm^{-1} M^{-1} at 290.5 nm and an absorbance coefficient of $\epsilon = 34.5$ cm^{-1} M^{-1} at 285 nm (Chen & Yang, 1977). Absorbance spectra were measured with a Cary 14 spectrophotometer or a Gilford Model 240 scanning spectrophotometer.

Stock solutions of H1 for CD measurement or for H1:DNA complexes were prepared by dissolving H1 at 0.4–1.0 mg/mL in water. The H1 concentration of the stock solution was determined by the procedure of Lowry et al. (1951) by using calf thymus H1 as a primary standard and bovine serum albumin (Nutritional Biochemicals) as a secondary standard. An absorbance coefficient of 1345 cm^{-1} M^{-1} was used for calf thymus H1 (Smerdon & Isenberg, 1976a). With the Lowry method, a 1.00-mg/mL solution of H1 has the same absorbance at 740 nm as an 0.87-mg/mL solution of bovine serum albumin. A mean residue molecular weight of 100.2 was calculated from the amino acid composition of calf thymus H1 (Smerdon & Isenberg, 1976a) and was used to calculate the CD of H1.

Salt solutions of H1 for CD measurement were prepared by adding a concentrated salt solution and 25 mM Tris-HCl (pH 7.2) to H1 in water. The final Tris-HCl concentration was 5 mM. The tube was closed and inverted several times. Most solutions were used for a single measurement; however, at low salt concentrations, the solutions were titrated with one or two additional aliquots. CD spectra of H1 (45 μg/mL) at different salt concentrations were measured in a 10-mm path length cell that had a 1.0-mL capacity.

Sedimentation Equilibrium Measurements. Sedimentation equilibrium measurements of H1 were performed at 20 °C with a Beckman Model E analytical ultracentrifuge as described by Yphantis (1964). A 12-mm charcoal-filled Epon centerpiece and an An-H Ti rotor were used for the measurements. The interference optics were focused at the two-thirds rd plane (Trautman, 1958), and the optical alignment was checked by the procedure of Dyson (1970). H1 was dissolved directly in 0.4 mL of sample buffer (0.10 or 0.50 M NaCl in 20 mM Tris-HCl, pH 7.2) and dialyzed against 50 mL of the same (4 °C) for 18 h. The final H1 concentration (0.5–0.8 mg/mL) was estimated from the absorbance at 220 nm after a tenfold dilution with water. The calculated specific volume (0.766 cm^3/g) of calf thymus H1 (Smerdon & Isenberg, 1976a) was used for molecular weight determinations. Solution densities were estimated from tables in the CRC Handbook of Chemistry and Physics (Weast, 1977).

Measurements on H1:DNA Complexes. H1:DNA complexes were prepared from $H1_0$ or $H1_P$ and PM2 phage DNA. PM2 DNA was isolated as described by Armel et al. (1977). The concentration of DNA stock solutions was determined from absorbance measurements and an absorbance coefficient of 20 cm^{-1} mL/mg at 260 nm. Boundary sedimentation measurements of the PM2 DNA at 20 μg/mL showed that it was composed of a faster sedimenting component (80%) and a slower sedimenting component (20%). Uncorrected sedimentation coefficients of the faster (30.9 ± 0.9 S) and slower components (21.1 ± 1.1 S) were essentially the same at NaCl concentrations of 7, 60, and 133 mM in 5 mM Tris-HCl, 0.3 mM EDTA (pH 7.2). The measured sedimentation coefficients of the fast component were about 7% higher than the values reported by Böttger & Kuhn (1971) for type I superhelical PM2 DNA, while the sedimentation coefficients for the slower component agreed with the previously reported values for type II nicked DNA (Böttger & Kuhn, 1971). H1 and PM2 DNA complexes for sedimentation boundary measurements were prepared by direct mixing in the sedimentation cell (Renz & Day, 1976). An aliquot of 150 μL of DNA solution (5 mM Tris, 0.3 mM EDTA, pH 7.2) was injected with a 250-μL Hamilton syringe into the sample sector of a Kel-F double-sectored cell. This was followed by 100 μL of an appropriate solution of NaCl in 5 mM Tris, 0.3 mM EDTA Finally, 150 μL of $H1_0$ or $H1_P$ in 5 mM Tris, 0.3 mM EDTA, 18 mM NaCl was injected. The sedimentation cells were shaken vigorously to mix the solution, which had a final DNA concentration of 20 μg/mL and an H1 concentration of 12 μg/mL.

Boundary sedimentation experiments of PM2 DNA and H1:DNA complexes were carried out in a Beckman Model E analytical ultracentrifuge equipped with an An-G Ti rotor at 22 ± 1 °C using only the refrigeration unit to control the temperature during the run. Sample absorbance was monitored at 265 nm, a wavelength at which DNA absorbs strongly, but the absorbance of H1 (12 μg/mL) is negligible. Therefore, the measurements monitor DNA concentration. The scanner stair-step optical density standards were used to calibrate absorbance which was read from the scanner chart. The relative concentrations of different sedimenting components were corrected for radial dilution (Schachman, 1959). Rotor speeds were raised sequentially from rest to 3000 rpm, to 6000 rpm, to 28 000 rpm. Complexes of $H1_P$:DNA and $H1_0$:DNA at a given salt concentration were compared in the same sedimentation run. Sedimentation coefficients are reported as measured and are not corrected to 20 °C with water as the solvent.

H1:DNA complexes for CD measurements were also prepared from $H1_0$ and $H1_P$ and PM2 DNA by direct mixing in the CD cell. H1 (0.34 mL) in 18 mM NaCl (5 mM Tris-HCl, 0.3 mM EDTA, pH 7.2) was added to 0.56 mL of PM2 DNA in NaCl solution (5 mM Tris-HCl, 0.3 mM EDTA, pH 7.2). The final DNA concentration was 40 μg/mL, while that of the H1 was 24 μg/mL. These concentrations are twice those used in the sedimentation boundary experiments. CD spectra were measured with the sample as close as possible (3.0 cm) to the phototube.

Results

Characterization of CHO H1. $NaDodSO_4$ electrophoresis of histone H1 from CHO and calf thymus (Figure 1) shows that the H1 preparations employed in this study are free of protein outside of the H1 mobility region and correspond to greater than 99.5% H1. Both CHO and calf thymus H1 (CT-H1) possess two sets of bands; however, the relative quantities in the bands are nearly reversed. About 75% of the CHO H1 is found in the faster band—in contrast to 35% for CT-H1. The functional significance of this type of subfraction variation is unknown. Perhaps it is involved with the variations in H1:HMG interactions reported by Smerdon & Isenberg (1976b).

Densitometer tracings of stained, long, acid–urea gels of CHO H1 from G_1-arrested cells and from cultures synchronously enriched in metaphase cells are shown in Figure 2. H1 from G_1-arrested cells is unphosphorylated (Gurley et al., 1975) and is designated $H1_0$. H1 from cultures synchronously enriched in metphase cells is a mixture of H1 molecules differing in their extent of phosphorylation and is designated $H1_P$. As previously shown (Gurley et al., 1978a),

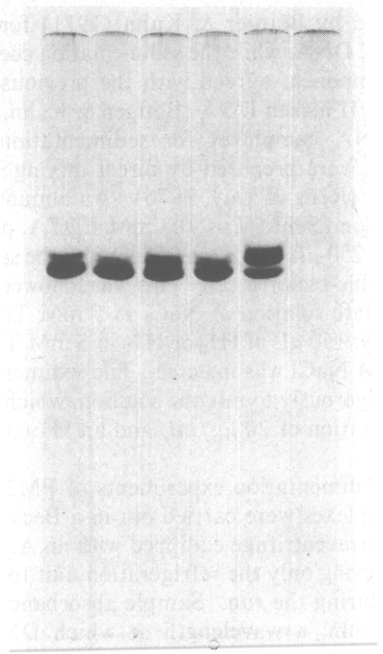

FIGURE 1: $NaDodSO_4$ gels of the H1 samples used for the physical measurements. From left to right: CHO $H1_P$, CHO $H1_0$, CHO $H1_{exp}$ from exponentially growing suspension cultures, another preparation of CHO $H1_{exp}$, CT-H1, and sample buffer. (Note: Stain at the origin of each gel does not originate with the H1 sample, since it is also present in the sample buffer gel.)

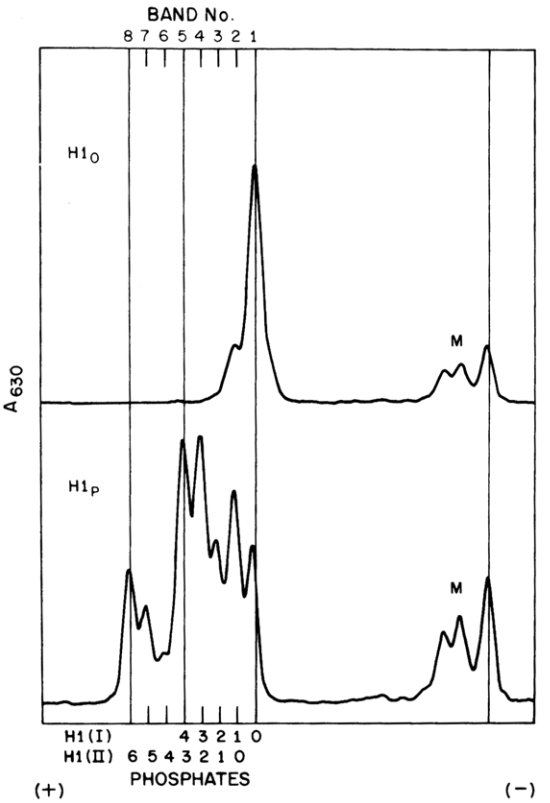

FIGURE 2: Electropherograms of CHO $H1_0$ and $H1_P$ from long (0.6 × 25 cm) acetic acid–urea polyacrylamide gels. The direction of migration is from left to right. The bands to the right and designated by M are those of CT-H1 that was loaded prior to CHO H1 as a mobility reference. H1(I) and H1(II) refer to the major and minor H1 subfractions that are found, respectively, in bands 1 and 2 of $H1_0$ (Hohmann et al., 1976).

H1 can be categorized into three classes differing in their extent of phosphorylation: (a) $H1_0$, which is unphosphorylated; (b) $H1_I$, having 1–3 phosphates per molecule; and (c) $H1_M$, having 4–6 phosphates per molecule. Classification of $H1_P$ indicates that 47% is $H1_M$, 41% is $H1_I$, and only 12% is $H1_0$. The average number of phosphates in $H1_P$ is estimated to be 2.9 per molecule. In a pure metaphase population, about 25% of the H1 has 5–6 phosphates per molecule, while 75% has 4 phosphates per molecule. In $H1_P$, about 19% has 5–6 phosphates per molecule, and about 28% has 4 phosphates per molecule. CHO H1 from exponentially growing cells ($H1_{exp}$) is 27% $H1_I$ and 73% $H1_0$ (gel trace not shown).

Like the spectrum of CT-H1 (Smerdon & Isenberg, 1976a), near-UV absorbance spectra of $H1_0$ and $H1_P$ in the 240–300-nm region are characteristic of tyrosine (ϵ = 1340 cm^{-1} M^{-1} at 275 nm). There is no sign of absorbance arising from adenine (ϵ = 13 400 cm^{-1} M^{-1} at 260 nm) that could be associated with ADP ribosylation of H1 (Hilz & Stone, 1976; Ord & Stocken, 1975); hence, any ADP ribosylation must make a negligible contribution to the phosphorylation of $H1_P$.

H1 Conformational Changes and Secondary Structure. Previous studies of the inner histones (Li et al., 1972; Wickett et al., 1972; D'Anna & Isenberg, 1974a–c; Smerdon & Isenberg, 1974; Bradbury et al., 1975b; Lewis et al., 1975; Pekary et al., 1975; Isenberg, 1977; Spiker & Isenberg, 1977) and of calf thymus H1 (Bradbury et al., 1975a; Smerdon & Isenberg, 1976a,b) have shown that acid-extracted histones undergo distinct conformational changes and interactions in solution. There is increasing evidence for the inner histones (D'Anna & Isenberg, 1974c; Sperling & Bustin, 1975; Weintraub et al., 1975; Camerini-Otero et al., 1976; Moss et al., 1976; Lewis, 1976; Bidney & Reeck, 1977) that histone complexes prepared from acid-extracted histones are the same as those complexes obtained directly from chromatin by salt extraction (Kornberg & Thomas, 1974; Roark et al., 1974). Thus, acid-extracted histones apparently can be renatured (Isenberg, 1977). In this section, we compare the salt-induced conformational changes of CHO $H1_0$ and $H1_P$ in solution with one another and with those of CT-H1. In addition to sodium chloride (Smerdon & Isenberg, 1976a), the divalent cation salts magnesium chloride and calcium chloride were also employed. Since it has been postulated that calcium may play a role in mitosis (Mazia, 1974; Harris, 1978) and since calcium is concentrated in chromosomes during mitosis (Cameron et al., 1977), it was of interest to see if divalent cations would alter the conformation of phosphorylated H1.

CD spectra of $H1_0$ and $H1_P$ in 1 mM HCl are identical with one another and with the spectrum of calf thymus H1 at the same conditions (Figure 3). The spectra are similar to those of the inner histones and are indicative of unordered polypeptides in solution (D'Anna & Isenberg, 1974a,b; Smerdon & Isenberg, 1976a). If the solution is 5 mM Tris-HCl (pH 7.2), the spectrum changes somewhat from the spectrum in 1 mM HCl; however, the spectrum (Figure 3) indicates that H1 remains essentially unordered in Tris buffer. Spectra of $H1_0$ and $H1_P$ are the same in 5 mM Tris-HCl. Upon addition of salt, H1 in all samples undergoes a rapid conformational change. CHO H1, like CT-H1 (Smerdon & Isenberg, 1976a), shows no evidence of the slow, time-dependent, conformational changes seen with the inner histones H3 and H4 (Li et al., 1972; Smerdon & Isenberg, 1973; D'Anna & Isenberg, 1974a).

CD measurements of $H1_0$ and $H1_P$ as functions of sodium chloride, magnesium chloride, and calcium chloride concentrations show that the induced conformational changes of $H1_0$ and $H1_P$ are the same in the respective salt solutions. In Figure 4, the quantity f, defined by eq 1 (Li et al., 1972; D'Anna & Isenberg, 1972), is plotted as a function of sodium

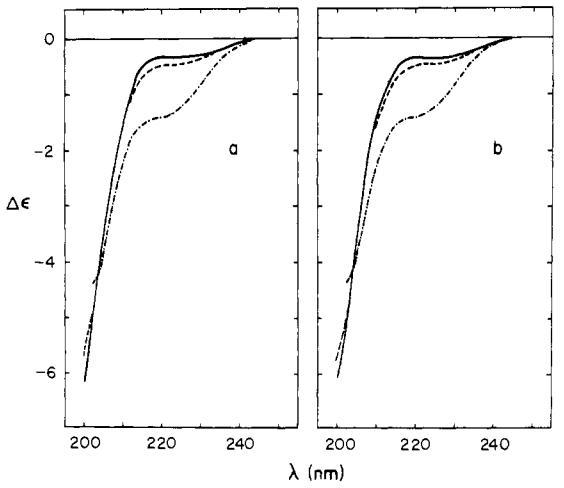

FIGURE 3: CD spectra of (a) CHO $H1_{exp}$ and (b) CT-H1. (—) H1 in mM HCl, (---) H1 in 5 mM Tris-HCl (pH 7.2), and (···) H1 in 0.40 M NaCl, 5 mM Tris (pH 7.2). Spectra were measured in a 2-mm path length cell at a concentration of 90 μg/mL. Since CHO $H1_0$, $H1_P$, and $H1_{exp}$ exhibited the same CD spectra at lower H1 concentrations, only $H1_{exp}$ is shown.

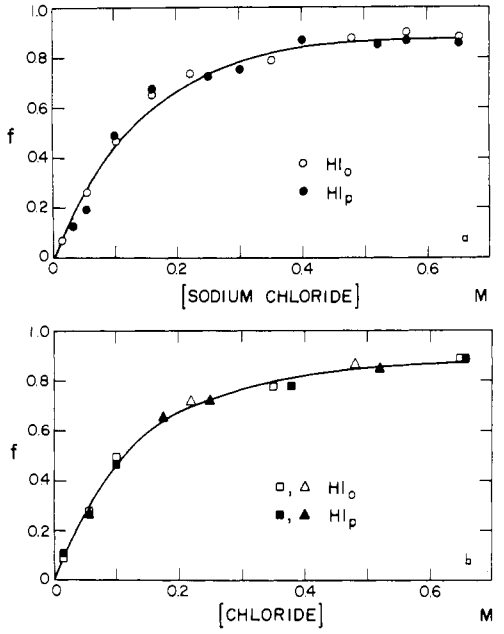

FIGURE 4: Functional dependence of the conformational changes of CHO $H1_0$ (open symbols) and CHO $H1_P$ (closed symbols) as functions of (a) sodium chloride concentration, and (b) calcium chloride (□; ■) and magnesium chloride (△; ▲) concentrations. Measurements were performed at 45 μg/mL in a 10-nm path length cell. The ordinate, f, is defined by eq 1 in the text.

chloride concentration (Figure 4a) or of chloride concentration for the chlorides of magnesium and calcium (Figure 4b). In eq 1, $\Delta\epsilon_{222}(P)$ is the circular dichroism measured at 222 nm

$$f = \frac{\Delta\epsilon_{222}(P) - \Delta\epsilon_{222}(0)}{\Delta\epsilon_{222}(\infty) - \Delta\epsilon_{222}(0)} \quad (1)$$

at the salt concentration [P], $\Delta\epsilon_{222}(0)$ is the circular dichroism at zero salt, and $\Delta\epsilon_{222}(\infty)$ is the value of $\Delta\epsilon$ obtained by extrapolation of $1/[\Delta\epsilon_{222}(P) - \Delta\epsilon_{222}(0)]$ vs. $1/[P]$ to the limit of [P] approaching infinity (Li et al., 1972; D'Anna & Isenberg, 1972). At $\Delta\epsilon_{222}(\infty)$, it is assumed that all H1 molecules will be in the folded conformation. From the definition of eq 1, it follows that f represents the fraction of change in CD of H1 in going from zero salt (unfolded H1) to the salt concentration [P], compared with the total conformational change in going from unfolded H1 to the fully folded conformation. The plots of Figure 4 clearly show that the conformational changes of $H1_0$ and $H1_P$ follow the same chloride functional dependence in either sodium chloride or the divalent cation chlorides. It is noted that CT-H1 is somewhat more sensitive to salt-induced conformational change than CHO H1. For example, 80 mM NaCl is required to induce one-half of the total conformational change in CT-H1 (data not shown); however, 120 mM NaCl (Figure 4a) is required to induce the same extent of conformational change in CHO H1. CD measurements of CHO $H1_P$ and CHO $H1_{exp}$ as functions of phosphate concentration (curves not shown) also indicate that the conformational changes of $H1_P$ and $H1_{exp}$ follow the same functional dependence in phosphate buffer.

Table I: Conformational Analysis[a] of the Circular Dichroic Changes in CHO and Calf Thymus H1 in Different Salt Solutions

sample	salt	$\Delta\epsilon_{222}(\infty)$[b]	% α	% β	% R	sum test
CHO $H1_P$	NaH_2PO_4	1.44	7.6	7.1	82.2	96.8
CHO $H1_{exp}$	NaH_2PO_4	1.44	7.6	7.1	82.2	96.8
CT-H1	NaH_2PO_4	1.48	6.2	8.6	85.2	100.0
CHO $H1_P$	NaCl	1.51	7.4	9.0	87.9	104.3
CHO $H1_0$	NaCl	1.58	7.9	9.6	87.1	104.6
CT-H1	NaCl	1.48	7.8	7.1	90.2	105.1
CHO $H1_P$	$CaCl_2$; $MgCl_2$	1.44	6.5	10.0	84.0	100.5
CHO $H1_0$	$CaCl_2$; $MgCl_2$	1.46	7.4	8.5	80.8	96.7
CT-H1	$CaCl_2$; $MgCl_2$	1.39	6.8	8.3	90.3	105.4

[a] Secondary structural analysis by the method of Baker & Isenberg (1976). The results are reported as percent α helix (% α), percent β sheet (% β), and percent random coil (% R). CD basis spectra used in the analysis were the α helix and β sheet of poly(L-lysine) and the random coil of the respective H1 in 1 mM HCl. All H1 random coils are the same. [b] CD value at 222 nm of the H1 molecule in the folded conformation. The value was obtained by extrapolation of a double inverse plot of the CD values and salt concentrations (Li et al., 1972).

We have interpreted the CD spectra by the method of Baker & Isenberg (1976), which was used for analyzing H1 subfractions (Smerdon & Isenberg, 1976a). The α helix and β sheet of poly(L-lysine) (Greenfield & Fasman, 1969) and the random coil of H1 in 1 mM HCl (Li et al., 1972; D'Anna & Isenberg, 1974a; Smerdon & Isenberg, 1976a) were used as basis spectra for the analysis in Table I. The net secondary structure of CHO $H1_0$, $H1_P$, $H1_{exp}$, and calf thymus H1 in their folded conformations is the same. About 15% of the H1 amino acid residues are contained in regions of α-helix and β-sheet structure (see Table I). These results agree with those of Smerdon & Isenberg (1976a) for the folded conformation of individual CT-H1 subfractions. We note that an equally acceptable analysis (Baker & Isenberg, 1976) can be obtained by employing the α-helix and β-sheet basis spectra of Chen et al. (1974) and the random coil of H1 in 1 mM HCl. These basis functions yield average values of 12.3 ± 0.6% α helix, 6.6 ± 0.9% β sheet, and 80 ± 4% random coil for all H1 preparations listed in Table I. The differences between the values of this analysis and those in Table I are probably within the limits of CD analyses to predict accurately the absolute quantities of secondary structure in largely unordered proteins (see Baker & Isenberg, 1976). Use of the α helix, β sheet, and random coil of poly(L-lysine) alone (Greenfield & Fasman, 1969) or the α helix, β sheet, and random coil of Chen et al. (1974) alone yields unacceptable analyses.

We conclude, therefore, that (a) the cell-cycle-dependent phosphorylations of $H1_P$ do not alter the sensitivity of CHO

Table II: Molecular Weights of H1 Samples Measured by High-Speed Sedimentation Equilibrium

sample	NaCl[a] (M)	$M_r \times 10^{-4}$ [b]
CHO $H1_0$	0.10	2.28
CHO $H1_P$	0.10	2.42
CT-H1	0.10	2.36
CHO $H1_P$	0.50	2.38
CT-H1	0.50	2.32

[a] The sodium chloride solutions were buffered with 20 mM Tris-HCl (pH 7.2). [b] The molecular weight (M_r) given in this column was calculated from the slope of a linear least-squares plot of ln c vs. $(r^2 - r_0^2)$, in which r is the distance from the center of rotation, c is the concentration in fringes at r, and r_0 is the distance from the center of rotation at the meniscus. Measurements were performed at 40 000 rpm.

H1 to undergo salt-induced folding; (b) the net secondary structure of the folded H1 is not affected by H1 phosphorylation; (c) the presence of divalent cations, which might bind to H1 phosphates, does not affect H1 folding or the folded conformation of H1; and finally (d) the net secondary structure of folded CHO H1 is the same as that of CT-H1 and involves about 15% of the H1 residues. While it is clear that divalent cations do not cause changes in the CD properties of $H1_P$ compared with those of $H1_0$, the measurements do not indicate whether or not divalent cations actually bind to $H1_P$ phosphate groups or if such binding could compete with divalent cation binding to DNA (Sander & Ts'o, 1971; Jacobs et al., 1976).

Sedimentation Equilibrium of H1. High-speed sedimentation equilibrium measurements (Yphantis, 1964) were performed to determine if phosphorylation induced dimerization or higher ordered aggregation of $H1_P$. Sedimentation equilibrium of $H1_P$, $H1_0$, or CT-H1 was measured at 0.10 M NaCl or 0.50 M NaCl in 20 mM Tris (pH 7.2). All solutions gave sedimentation results consistent with H1 being a monomer species having a molecular weight of $2.35 \pm 0.05 \times 10^4$ (Table II). There is no evidence that H1 phosphorylation induces H1 dimerization in solution. Plots of weight-average molecular weight vs. H1 concentration (not shown) give slightly negative slopes, indicating nonideality of H1 in the solutions.

These results for CHO H1 and CT-H1 agree with other measurements of calf thymus (Smerdon & Isenberg, 1976a) and indicate that H1 is monomeric at concentrations up to 1.5 mg/mL. The weak monomer–dimer equilibrium of CT-H1 measured by Roark et al. (1976) for H1 obtained from salt extracts of chromatin is not observed.

H1:DNA Complexes. There have appeared a number of model studies of H1 binding to DNA (Böttger et al., 1976; Renz & Day, 1976; Singer & Singer, 1976) or H1 binding to oligonucleosomes initially depleted of H1 (Renz et al., 1977). H1 binds cooperatively to linear DNA (Renz & Day, 1976) and to oligonucleosomes depleted of histone H1 (Renz et al., 1977); the cooperativity is dependent on both DNA size (oligonucleosome length) and sodium chloride concentration. It has also been observed that H1 binds preferentially to naked, closed, circular, superhelical DNA, compared with relaxed, closed, circular DNA (Singer & Singer, 1976), and that H1 binding to superhelical DNA leads to the formation of very large aggregates (Böttger et al., 1976). We examine here the boundary sedimentation of H1:DNA complexes prepared from CHO $H1_0$ or CHO $H1_P$ with closed, circular PM2 DNA [80% superhelical (type I); 20% nicked, closed, circular (type II)]. Complexes were prepared at a histone:DNA ratio of 0.6:1.0, and they were examined as a function of sodium chloride concentration (Renz & Day, 1976).

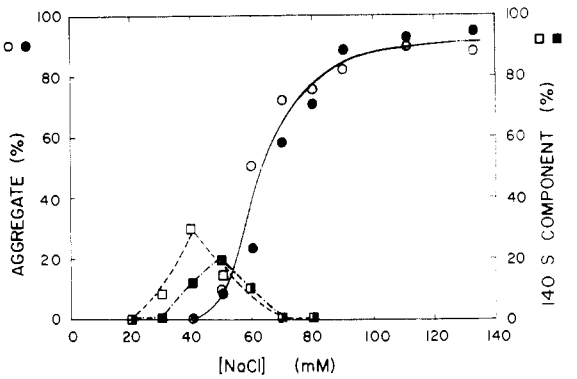

FIGURE 5: Boundary sedimentation results for H1:DNA complexes: the 140S component of $H1_0$:DNA (□); 140S component of $H1_P$:DNA (■); large aggregate of $H1_0$:DNA (○); and large aggregate of $H1_P$:DNA (●). The aggregate refers to the heterogeneous material with a sedimentation coefficient greater than 1500 S.

Three classes of sedimenting species were observed in H1:DNA mixtures: (a) a component having a sedimentation coefficient of 31.8 ± 1.0 S; (b) a component with a sedimentation coefficient of 140 ± 20 S; and (c) heterogeneous aggregates having sedimentation values ≥1500 S. At 20 mM NaCl, all DNA in the mixtures sedimented at 32 S (the sedimentation coefficient of naked type I DNA); even the 20% type II (which alone had a 21S sedimentation coefficient) sedimented at 32 S. Therefore, while it is not known if H1 is bound to the type I DNA at 20 mM NaCl, it surely is bound to all type II because no 21S component was observed. As the salt concentration was raised, the 140S component and heterogeneous aggregates were observed in addition to the 32S component. The appearance and quantitation of the 140S component and of the aggregates are shown in Figure 5.

While $H1_0$ and $H1_P$ both induce formation of the 140S component, $H1_0$ induces the 140S component at a slightly lower sodium chloride concentration than does $H1_P$. In both cases, however, the 140S component begins to disappear with the formation of the large aggregates. Induction of the aggregates is sigmoidal with respect to salt concentration, and data points for the $H1_P$:DNA and $H1_0$:DNA aggregates are within experimental error of the sigmoidal curve of Figure 5. Therefore, while the data show that $H1_0$ is somewhat more effective than $H1_P$ for inducing the 140S component, the difference in the required salt concentration is ≤10 mM. These data qualitatively are very similar to those measured by Renz & Day (1976) for H1:DNA complexes prepared with unphosphorylated CT-H1 and sheared CT-DNA.

CD spectra of the $H1_0$:DNA and $H1_P$:DNA complexes were measured at 20, 40, and 120 mM NaCl (Figure 6) to determine if H1 phosphorylation altered the symmetry of DNA in the H1:DNA complexes. At both 20 and 40 mM NaCl, the CD spectra of the $H1_0$:DNA and $H1_P$:DNA samples are the same; however, the spectra of the complexes are different from that of the DNA control. Absorbance spectra of the complexes at 20 and 40 mM NaCl have A_{350nm}/A_{260nm} ratios of ≤0.015. This indicates that there is negligible light scattering of the samples, which is consistent with the sedimentation data of Figure 5. At 120 mM NaCl where large aggregates are formed (Figure 5), the CD spectra of the complexes (Figure 6c) are different from one another and from that of the DNA control. The complexes also exhibit considerable turbidity ($A_{350nm}/A_{260nm} = 0.12$), and the measured CD spectra vary slightly with the distance of the CD cell from the phototube. The latter observation indicates that differential scattering of the left and right circularly polarized light is contributing to the measured CD spectra (Dorman & Maestre,

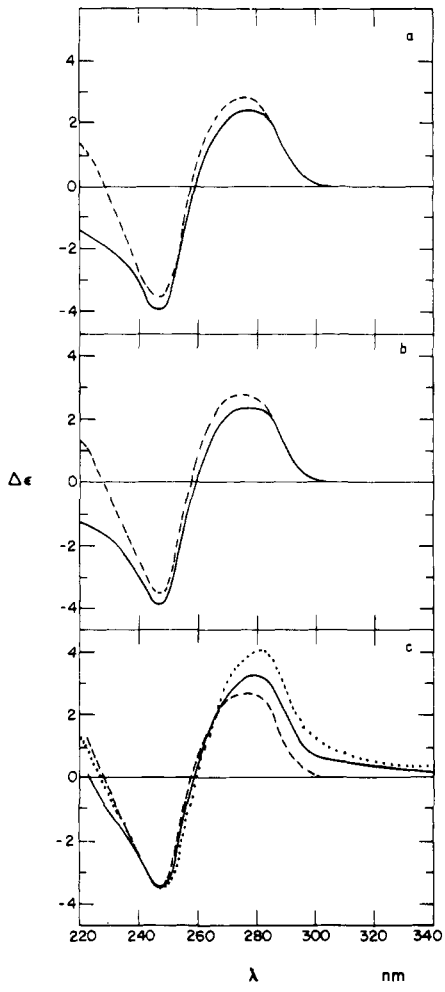

FIGURE 6: CD spectra of H1:DNA complexes prepared from $H1_0$ or $H1_P$ and PM2 DNA. (a) Spectra in 20 mM NaCl: (---) DNA, (—) $H1_0$:DNA and $H1_P$:DNA. (b) Spectra in 40 mM NaCl: (---) DNA; (—) $H1_0$:DNA and $H1_P$:DNA. (c) Spectra in 120 mM NaCl: (---) DNA; (—) $H1_P$:DNA; (···) $H1_0$:DNA. Spectra were measured in a 10-mm path length cell containing 0.90 mL of solution. H1 is 24 μg/mL and DNA is 40 μg/mL.

1973). Therefore, at 120 mM NaCl, $H1_0$ and $H1_P$ differ in their interaction with DNA so that the circular dichroism of the DNA in the aggregates is altered and/or the symmetry of the aggregates themselves is altered.

Adler et al. (1972) also observed differences between the CD spectra of H1:DNA complexes prepared from in vitro phosphorylated H1 or unphosphorylated H1 with linear CT-DNA. Since their complexes employed linear DNA and the complexes were prepared by stepwise gradient dialysis, detailed comparisons are not possible.

Discussion

We have reported what we believe to be the first investigation of some physical properties of H1 phosphorylated in vivo. The phosphorylated material, $H1_P$, does not represent a pure phosphorylation state of H1, but it does contain a defined composition of naturally phosphorylated H1 molecules [47% having 4–6 phosphates per molecule ($H1_M$), 41% having 1–3 phosphates per molecule ($H1_I$), and 12% having unphosphorylated $H1_0$]. Also, the average number of 2.9 phosphates per molecule exceeds the 2.2 phosphates per molecule in CT-H1 enzymatically phosphorylated in vitro with growth-associated kinase (Matthews & Bradbury, 1978).

CD measurements of CHO $H1_0$, $H1_P$, and $H1_{exp}$ were identical with one another. Since $H1_P$ was almost 50% $H1_M$ and 40% $H1_I$, it is clear that neither mitotic nor interphase levels of phosphorylation altered the CD properties of H1. These same measurements also indicate that the net secondary structure of CHO H1 is the same as that of CT-H1. The latter result is consistent with sequence data (MacLeod et al., 1977) and proton magnetic resonance (^{1}H NMR) studies of Hartman et al. (1977). Sequence studies (MacLeod et al., 1977) show that the most highly conserved region, when trout H1 is compared with rabbit H1, is the 43–112-residue region of rabbit H1. It is this region in CT-H1 that has been identified from ^{1}H NMR studies (Hartman et al., 1977) as being folded. If it is assumed that this region is conserved in both CHO H1 and CT-H1, then the secondary structures should be similar. We note, however, that the sensitivity to folding of whole CHO H1 differs from that of whole CT-H1; furthermore, Smerdon & Isenberg (1976a) have observed that even CT-H1 primary-structure subfractions differ in their sensitivity to folding in salt solutions. It is not known if these differences arise from amino acid substitutions directly in the regions of secondary structure or at other positions that induce subtle differences in tertiary structure. Regardless, it does not appear that H1 phosphorylation is affecting H1 folding or the net secondary structure of the folded conformation.

Sedimentation equilibrium measurements at 0.10 and 0.50 M NaCl show that phosphorylated CHO $H1_P$, like CHO $H1_0$ and CT-H1, is a monomer in solution. It should be considered, however, that, since H1 is highly basic, positive charge repulsions between H1 molecules in solution could prevent dimer formation or cooperative interactions (Renz et al., 1977) that might otherwise occur in chromatin.

Although H1 phosphorylation has been temporally associated with condensed states of chromatin [see Gurley et al. (1978b) for a review], $H1_0$, rather than $H1_P$, induces the 140S H1:DNA complex at a slightly lower salt concentration than does $H1_P$. However, there is no significant difference between $H1_0$ and $H1_P$ in inducing the large H1:DNA aggregates. If H1 phosphorylation were stabilizing higher orders of chromatin structure and if H1:DNA complexes were suitable models, then phosphorylation of $H1_P$ might be expected to shift the equilibria so as to induce aggregate formation at lower salt concentration than that required by $H1_0$. This was not observed. Hence, these results suggest that either phosphorylation plays a limited role in mechanically inducing or stabilizing chromatin condensation or that H1:DNA complexes are incomplete models for H1 interactions in chromatin. Other data in the literature support the notion that H1 phosphorylation is unnecessary for H1 to serve a condensing function. For example, while Matthews & Bradbury (1978) have observed increases or decreases in the turbidity of H1:DNA complexes depending on the sites of in vitro H1 phosphorylations, aggregated complexes were formed with *all* H1 samples. Others also have shown that unphosphorylated H1 can induce the formation of aggregated H1:DNA complexes (Böttger et al., 1976; Renz & Day, 1976) and that unphosphorylated H1 can induce solenoid formation (Finch & Klug, 1976) or compaction (Renz et al., 1977) of oligonucleosomes. A direct comparison of the chemical and physical properties of isolated oligonucleosomes containing the fully superphosphorylated H1 with those of isolated oligonucleosomes containing the unphosphorylated $H1_0$ would be desirable to help clarify these points. Such comparisons are complicated, however, because the $H1_M$ phosphorylations are largely depleted during the isolation of metaphase chromosomes by standard procedures (D'Anna et al., 1978).

Since completion of this work, two additional studies employing in vitro phosphorylated H1 have appeared. Singer

& Singer (1978) have studied in detail the binding of unphosphorylated and phosphorylated CT-H1 samples with SV40 DNA. Knippers et al. (1978) have studied the binding of in vitro phosphorylated ascites H1 and in vitro phosphorylated lymphocyte H1 with Col E1 DNA. With regard to H1 binding to superhelical DNA, both studies find approximately equal binding of phosphorylated or unphosphorylated H1 to superhelical DNA at 100 mM NaCl; however, they find that phosphorylated H1 binds more efficiently than unphosphorylated H1 in the absence of salt or at very low ionic strengths (20–40 mM NaCl). Knippers et al. (1978) have suggested that, at 20 mM NaCl, phosphorylation reduces the ability of H1 to bind cooperatively to DNA. We cannot distinguish differential binding of H1 to DNA in our sedimentation boundary experiments; however, the CD spectra of H1:DNA complexes of $H1_0$ or $H1_P$ are the same at 20 and 40 mM NaCl. Therefore, if differential binding is occurring, it is not affecting the CD spectra. Singer & Singer (1978) have also found that unphosphorylated H1 and superhelical DNA mixtures undergo a salt-dependent transition ("between 40 and 100 mM NaCl") to form aggregated H1:DNA complexes. Similar data for their phosphorylated H1 samples are not available for comparison with our results in Figure 5.

We point out that unfractionated CHO H1 was employed in these studies. CHO H1, however, appears to contain a number of primary-structure subfractions (Gurley et al., 1975), like H1 from other species (Kincade & Cole, 1966; Bustin & Cole, 1969; Langan et al., 1971; Stout & Phillips, 1973; Sherod et al., 1974; Smerdon & Isenberg, 1976a; Spiker, 1976). While our measurements of unfractionated CHO H1 provide no evidence that mitotic phosphorylation of H1 alters H1 conformation or H1:H1 interactions, small conformational changes or weak dimer formation by minor H1 subfractions may not have been detected. If such small, subfraction-specific, conformational changes are induced by phosphorylation, their detection will require the isolation of each phosphorylated subfraction and its unphosphorylated counterpart.

In summary, these measurements of CHO H1 phosphorylated in vivo indicate that H1 phosphorylation does not affect H1 secondary structure, it does not induce H1:H1 interactions in solution, and it does not greatly affect the sedimentation properties of H1:DNA complexes. Phosphorylation, however, does alter the interaction of H1 with DNA at 120 mM NaCl where the H1:DNA complexes aggregate. These findings, therefore, minimize a number of possible effects of CHO H1 phosphorylation and focus attention on other possible roles of H1 phosphorylation in chromatin structure. It seems more likely that (a) CHO H1 phosphorylations may alter the binding of specific regions of H1 to the DNA of internucleosomal spacer regions (or to nucleosomes); or (b) CHO H1 phosphorylations may modulate the H1:HMG interactions observed by Smerdon & Isenberg (1976b) and/or the interaction of H1 with other chromosomal proteins. Glotov et al. (1977) recently have reported that phosphorylation at serine-37 [which does not appear to occur in CHO cells (Hohmann et al., 1976)] alters H1:DNA binding so that the tyrosine-containing region of H1 becomes less rigidly bound in H1:DNA complexes. Matthews & Bradbury (1978) also have alluded to similar observations for H1 phosphorylated with the "growth-associated" kinase. Whether or not similar effects occur with the cell-cycle-related phosphorylations considered in this report remains to be determined.

Acknowledgments

We thank Dr. Robert A. Tobey for his advice in synchronizing CHO cells and Joseph G. Valdez, Linda Ferzoco, and Rubanna Garcia for their excellent technical assistance during this research.

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