system the retention volumes (mL) of the various products were: ox-redC, 11; ox-redA, 13; A(MeOEt), 16; cordycepin, 19; A, 32; A(MeOEt)A(MeOEt), 45; A2′p, 69; A3′p, 74; pAp, 92. In the case of the analyses of oligonucleotides containing *ara*-adenosine moieties a similar analytical chromatographic system was used except that the elution solvent contained 40% ethanol. The corresponding retention volumes (mL) in this system were: *ara*-A, 33; *ara*-A-*ara*-A, 41; Ap, 61; pAp, 84.

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Characterization of Complexes of Superhelical and Relaxed Closed Circular DNA with H1 and Phosphorylated H1 Histones†

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ABSTRACT: Complexes of H1 histone and closed circular SV40 DNA have been analyzed on sedimentation velocity gradients. At 100 mM NaCl, the binding of H1 histone to relaxed DNA is cooperative, resulting only in rapidly sedimenting complexes. These complexes consist of an average of 40–45 molecules of H1 histone per molecule of relaxed DNA. Under the same conditions, the interaction of H1 histone with superhelical DNA yields two distinct types of complexes: a slowly sedimenting complex and a rapidly sedimenting complex. The slowly sedimenting complex contains an average of 10–12 H1 histone molecules per molecule of superhelical DNA. The rapidly sedimenting complex arises as a result of the cooperative binding of H1 histone to superhelical DNA; the number of H1 histone molecules per superhelical DNA molecule in this complex ranges from 45 to 80. The cooperative binding of H1 histone to DNA is salt sensitive. It is further demonstrated that, of four different species of phosphorylated H1 histone tested, all bind DNA and retain the ability to discriminate between superhelical and relaxed DNA with the same efficiency as the nonphosphorylated H1 histone. Sedimentation velocity gradient analysis of the complexes of one species of phosphorylated H1 histone and circular DNA indicates that, although the nature of the binding is qualitatively similar to that of nonphosphorylated H1 histone, there are quantitative differences.

The chromatin of eukaryotes is organized in a linear array of structural subunits called nucleosomes (Oudet et al., 1975; Olins & Olins, 1974). Nucleosomes consist of a core particle containing two each of the four histone classes H2A, H2B, H3, and H4 closely associated with 140 base pairs of DNA and less tightly associated with approximately 60 additional base pairs of DNA (Kornberg, 1974; Van Holde et al., 1974; Hewish & Burgoyne, 1973; Sollner-Webb & Felsenfeld, 1975; Noll, 1974; Simpson & Whitlock, 1976). Physical studies on nucleosome structure demonstrate that the DNA of the nucleosome is wound around the outside of the protein core (Baldwin et al., 1975) in a manner which is topologically equivalent to a supercoiling of the DNA (Germond et al., 1975).

The structural relationship of the fifth major class of histones, the very lysine-rich H1 histones, to this fundamental unit of chromatin is not known. H1 histone has been reported to be associated with the 60 base pair segment of nucleosomal DNA (Varshavsky et al., 1976) and with regions of internucleosomal DNA (Noll & Kornberg, 1977). H1 histone may induce higher order packaging of nucleosomes.

H1 histone is distinct from the other four histones in a number of ways. Calf thymus H1 histone, a protein of mol wt

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TABLE I: Phosphorylated Calf Thymus H1 Histone Preparations.[a]

Type of H1	Amino acid residue phosphorylated	Phosphate content[b] (mol of P/mol of H1 histone)
H1(A)	Serine-37	0.86
H1(A) control[c]		0.15
H1(B)	Serine-106	1.22
H1(B) control		0.20
H1(AB)	Serines-37 and -106	1.98
H1(AB) control		0.24
H1(GA)	Threonine-16, serine-156, 3 other sites[d]	2.31
H1(GA) control		0.19
[^{32}P]H1(AB)	Serines-37 and -106	1.52

[a] These materials were prepared and characterized by Dr. Thomas A. Langan. [b] Estimated by release of free phosphate following treatment of sample with alkaline phosphatase. [c] Control histones were incubated in the phosphorylation system in the absence of ATP. [d] These sites are only tentatively identified. The extent to which any of these sites is phosphorylated in this sample is unknown.

21 000 composed of 212 amino acid residues, is highly asymmetric in both the distribution of basic amino acid residues and its secondary structure (Bustin & Cole, 1969; Bradbury et al., 1975). The molecule has a short highly basic tail at the amino-terminal end and a globular central core region containing a relatively high proportion of the hydrophobic amino acid residues; the carboxy-terminal half of the molecule consists largely of alanine, proline, and lysine residues. Unlike the other four histones which are highly conserved in evolution, the primary sequence of H1 histone shows extensive interspecies and interorgan variation as a result of both conservative and nonconservative amino acid replacements in the basic regions of the molecule (DeLange & Smith, 1971). The central globular region, however, remains largely invariant (R. D. Cole, personal communication).

H1 histone can be modified through phosphorylation (Langan & Hohmann, 1975). Changes in the extents of phosphorylation of H1 histone have been correlated with the onset of both mitosis (Lake & Salzman, 1972) and cellular DNA synthesis (Balhorn et al., 1972). It has also been proposed that one type of phosphorylation of H1 histone serves as a trigger for the condensation of chromosomes during mitosis (Bradbury et al., 1974). Site-specific phosphorylation of H1 histone occurs in rat liver following administration of the hormone glucagon (Langan, 1969a).

Phosphorylation of H1 histone occurs at a number of distinct serine and threonine sites (Langan & Hohmann, 1975). One site, in the amino-terminal tail of the molecule (serine-37), is phosphorylated by a cAMP-dependent protein kinase in response to glucagon stimulation (Langan, 1969b). A second site in the globular core region (serine-106, adjacent to the single phenylalanine) can be phosphorylated in vitro by a cAMP-independent histone kinase (Langan, 1971), although in vivo phosphorylation of this site has not yet been demonstrated. Another set of five sites, distributed throughout the H1 histone molecule, is phosphorylated during periods of rapid cell growth (Langan & Hohmann, 1974). It has been proposed that phosphorylation at these various sites may alter histone–DNA interactions (Adler et al., 1972).

Earlier studies from this laboratory indicated that H1 histone from a variety of sources and the avian very lysine-rich histone, H5, complex superhelical DNA more efficiently than relaxed, closed circular DNA, as measured by a nitrocellulose filter binding assay (Vogel & Singer, 1975a,b, 1976; Bina-Stein et al., 1976; Singer & Singer, 1976). The other four major classes of histones do not distinguish between the relaxed and superhelical forms of DNA by this assay. Studies of various polypeptide fragments of the H1 histone molecule indicated that the globular region of the calf thymus H1 histone molecule is involved in the discrimination between superhelical and relaxed DNA (Singer & Singer, 1976). The binding of H1 histone does not alter the net superhelical density of a closed, circular DNA molecule, and therefore probably does not unwind the DNA duplex (Bina-Stein & Singer, 1977).

We have now extended our earlier work by determining the types of complexes formed and the stoichiometry of binding of H1 histone to supercoiled and relaxed DNA. The effect of phosphorylation at various sites on the H1 histone molecule on the ability to bind and discriminate between the two forms of circular DNA has also been examined.

Experimental Procedures

All DNA preparations were simian virus 40 DNA labeled with [^{14}C]thymidine or [^{3}H]thymidine, as indicated in the legends to the figures. Closed, circular duplex superhelical DNA (DNA I)[1] and closed circular duplex relaxed DNA (DNA I^{r}) were prepared as described previously (Bina-Stein & Singer, 1977). Specific radioactivities were as indicated in the legends to the figures.

Calf thymus H1 histone was obtained from Worthington Biochemical Corp. and labeled with tritium by New England Nuclear Corp. Following the labeling procedure, H1 histone was purified in this laboratory by two successive gel filtrations through a 1.9 × 40 cm column of Bio-Gel P-60. Column elution was as described previously (Bohm et al., 1973; Singer & Singer, 1976). Following purification, there was no remaining exchangeable tritium. The concentration of the [^{3}H]H1 histone stock solution was determined by amino acid analysis to be 287.4 μg/mL. The specific radioactivity of the purified [^{3}H]H1 histone was 2.55×10^{5} cpm/μg. Immediately following purification, analysis of this material by gel electrophoresis gave only the expected double band of protein characteristic of calf thymus H1 histone. Fluorography of the gels (Bonner & Laskey, 1974) showed that all detectable radioactivity was associated with these two bands. However, after prolonged storage at −20 °C, small amounts of degraded material could be observed. This was most likely due to radiation damage. [^{3}H]H1 histone exhibited the expected specificity for supercoiled DNA relative to relaxed DNA in the nitrocellulose filter binding assay (Vogel & Singer, 1975a) (data not shown). Preparation of the carboxy-terminal fragment containing amino acid residues 107–212 of H1 histone was as described before (Singer & Singer, 1976).

All phosphorylated H1 histone samples and nonphosphorylated controls were prepared from calf thymus according to the methods of Langan (1978) and were a gift from Dr. Thomas A. Langan (University of Colorado, Denver). The extent and sites of phosphorylation are indicated in Table I.

The concentration of the ^{32}P-labeled H1(AB) histone was determined directly by amino acid analysis; its specific ra-

[1] Abbreviations used: DNA I, superhelical DNA; DNA I^{r}, relaxed, closed circular DNA; S complex, slowly sedimenting complex; P complex, rapidly sedimenting complex; H1(A) histone, H1 histone phosphorylated at serine residue 37; H1(B) histone, H1 histone phosphorylated at serine residue 106; H1(AB) histone, H1 histone phosphorylated at both residues, 37 and 106; H1(GA) histone, H1 histone phosphorylated at 2–3 of five positions; BSA, bovine serum albumin; cAMP, cyclic adenosine 3′,5′-monophosphate.

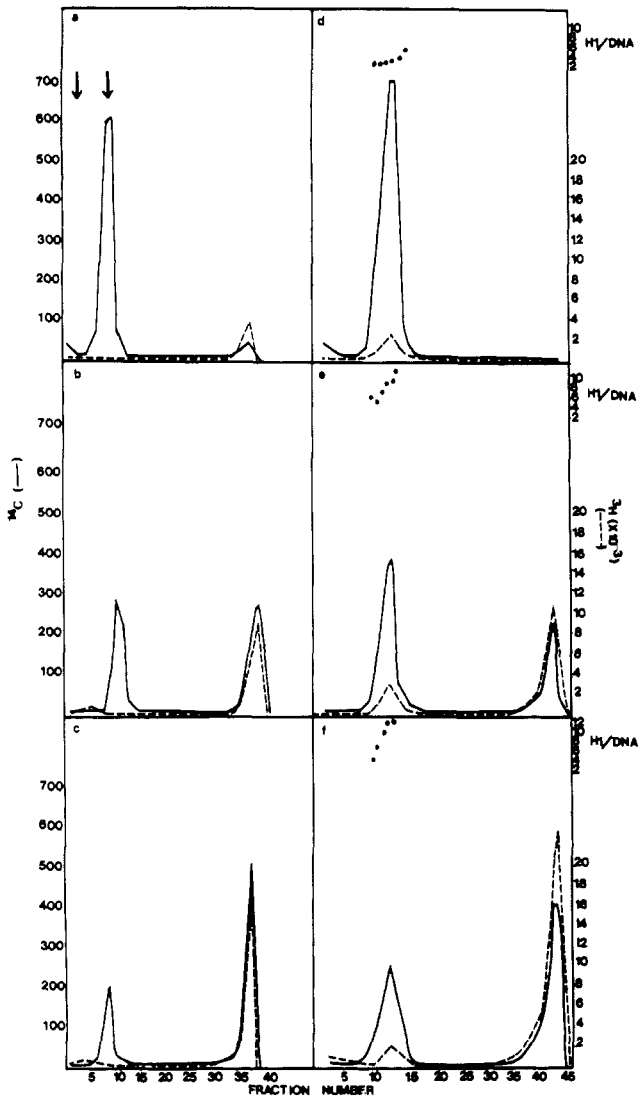

FIGURE 1: Sedimentation velocity gradient profiles of complexes of H1 histone with DNA. [^{3}H]H1 histone·[^{14}C]DNA complexes were formed at different R values and separated on 5–20% metrizamide gradients as detailed in the text. Sedimentation was from left to right. The arrows mark the positions of free H1 histone and free DNA. Specific activities were: [^{14}C]DNA I, 1798 cpm/μg; [^{14}C]DNA I^r, 2116 cpm/μg. (a) H1 histone·DNA I^r, R = 11.5; (b) H1 histone·DNA I^r, R = 38.8; (c) H1 histone·DNA I^r, R = 42.2; (d) H1 histone·DNA I, R = 6.6; (e) H1 histone·DNA I, R = 24.2; (f) H1 histone·DNA I, R = 34.7. In panels d–f, the molar ratio of H1/DNA I of each fraction in the S complex, calculated as described in Experimental Procedures, has been plotted above the S complex peak. [^{14}C]DNA (—); [^{3}H]H1 histone (- - -).

dioactivity was determined for each experiment and is indicated in the legends to the figures.

The concentrations of solutions of unlabeled histone samples were estimated from the absorption at 230 nm, assuming an absorbance of 4.25 for a 1 mg/mL solution (Ohlenbusch et al., 1967). However, our own amino acid analyses indicated that this absorbance value is an overestimate. Recently, Renz & Day (1976) reported an extinction coefficient at 230 nm of 2.0 for a 1 mg/mL solution. Therefore, R values (see below) for the experiments with unlabeled histones (Figures 3, 6, and 7) may not be comparable to those where radioactive H1 histone was used.

Methods. Filter Binding Assay. Interaction between DNA and H1 histone samples was measured by determining the protein-dependent accumulation of [^{14}C]DNA on nitrocellulose filters, as described previously (Vogel & Singer, 1975a; Singer & Singer, 1977). The results are expressed as the percentage of input counts of [^{14}C]DNA retained on the filter as a function of the R value. The R value is defined as the molar input ratio of protein to DNA (expressed as molecules of DNA, not nucleotides). Molecular weights of 21 000 for H1 histone and 3.3×10^6 for SV40 DNA were used to calculate both the R value and the molar ratio of binding in complexes (see below). In all cases, the concentration of DNA used in the filter binding assays was approximately 1 μg/mL; the H1 histone concentration ranged from 0.01 to 2.0 μg/mL.

TABLE II: Molar Ratio of H1 Histone/DNA I^r in Complexes.

R value	H1 histone in P complex (%)	DNA I^r in P complex (%)	Molar ratio of H1/DNA I^r in P complex
6.3	76	5	27
11.5	92	11	41
19.9	91	23	36
38.8	95	50	44
42.4	96	67	45
68.0	98	96	51

Complex Formation. A constant amount of radioactively labeled DNA was incubated with varying amounts of differentially labeled H1 histone in 100 mM NaCl, 50 mM Tris-HCl, pH 7.8, 1 mg/mL BSA, 1 mM EDTA (standard binding buffer) for 15 min at 23 °C. In some experiments indicated in the text, the NaCl concentration was varied between 0 and 200 mM. At the end of the incubation aliquots were removed to determine the concentration of reactants in the mixture.

Sedimentation Velocity Gradient Analysis. The remainder of the sample was layered on a 4.5-mL gradient of from 5 to 20% metrizamide formed on top of a 0.2-mL cushion of 75% metrizamide. Routinely, metrizamide (Accurate Chem. Co.) solutions were made in 100 mM NaCl, 50 mM Tris-HCl, pH 7.8, 1 mM EDTA. For the studies on the effect of NaCl concentration, the metrizamide solutions were made in 50 mM Tris-HCl, pH 7.8, 1 mM EDTA at the appropriate NaCl concentration. Cellulose nitrate centrifuge tubes were coated with 0.1% gelatin. In the absence of gelatin coating, recovery of [^{3}H]H1 histone following centrifugation was poor (20–30%). With gelatin coated tubes, recovery of [^{3}H]H1 histone was routinely 70–80%. Recovery of DNA was 90–100%. Gradients were centrifuged for 1 h at 20 °C at 42 000 rpm in a SW50.1 rotor of the Beckman L5-65 ultracentrifuge. Five-drop fractions were collected from the bottom of the tube and counted in a Triton–toluene based scintillation fluid. Spill-over of ^{14}C into the tritium channel was determined for each experiment and ranged between 18 and 19%. Spill-over from the tritium channel to the ^{14}C channel was less than 1%. Spill-over of ^{32}P into the tritium channel was 2.9% in all cases. Spill-over from the tritium channel to the ^{32}P channel was negligible. Only tritium counts were corrected for spill-over.

Calculation of Molar Ratio of H1 Histone to DNA in Complexes. The molar ratios of H1 histone to DNA in complexes were calculated from either (a) total number of [^{32}P]-H1(AB) histone and [^{3}H]DNA counts or (b) total number of [^{3}H]H1 histone and [^{14}C]DNA counts recovered in a gradient peak, using the specific radioactivities and molecular weights given above.

Results

H1 Histone·DNA I^r Complexes. Complexes were formed by combining [^{3}H]H1 histone and ^{14}C-labeled DNA I^r at various R values (at a constant DNA concentration) and then

TABLE III: Molar Ratio of H1 Histone/DNA I in Complexes.

	Slowly sedimenting complex (S complex)[a]			Rapidly sedimenting complex (P complex)		
R value	H1 histone in S complex (%)	DNA in S complex (%)	Molar ratio of H1/DNA I	H1 histone in P complex (%)	DNA in P complex (%)	Molar ratio of H1/DNA I
4.6	96	99	3	3	1	
6.6	92	98	4	7	1	
15.3	52	87	7	47	12	47
24.2	29	68	9	70	31	49
31.1	22	63	9	77	36	54
34.7	13	45	10	86	54	56
45.7	8	33	11	91	66	63
51.8	5	20	13	94	79	62
71.6	2	3		98	96	76

[a] The definition of the S complex is based solely on its position in a velocity gradient, which is indistinguishable from that of free DNA. Since the fraction of the S complex which is free DNA is not known, the values given are representative of the percentage of the total DNA which is recovered in the slowly sedimenting peak, i.e., a mixture of free and histone-bound DNA. Similarly, the calculated molar ratios must be viewed as average values.

characterized on sedimentation velocity gradients. Under the conditions used, H1 histone remained near the top of the gradient in the absence of DNA; free DNA sedimented to a position approximately one-quarter of the length of the gradient from the top (Figure 1a). The sedimentation profiles of complexes formed at various *R* values are shown in Figure 1 (a–c). At low *R* values, most of the DNA I^r sedimented in the same position as free DNA I^r. However, a small fraction of the DNA I^r, associated with most (90–92%) of the H1 histone, was found floating on the cushion at the bottom of the gradient. With increasing *R* values, an increasing proportion of the DNA I^r sedimented onto the cushion as a rapidly sedimenting H1 histone·DNA I^r complex (P complex) (Table II). Above an *R* value of 42, all of the DNA I^r was recovered as a rapidly sedimenting complex. No complexes of intermediate sedimentation velocity were observed at any of the ratios tested; nor was H1 histone ever observed associated with DNA I^r at the position of free DNA.

At all *R* values including the highest tested, nearly all of the H1 histone was recovered in the P complex. However, approximately 8–10% of the [^{3}H]H1 histone was recovered as unbound material at the top of the gradient at all *R* values. The proportion of this unbound material remained constant over the range of *R* values examined. This material probably consisted of degradation products of the [^{3}H]H1 histone sample as indicated by sodium dodecyl sulfate gel analysis (data not shown).

From the specific radioactivities of the [^{3}H]H1 histone and [^{14}C]DNA I^r, the average number of H1 histone molecules bound per molecule of DNA I^r in the P complex was calculated; these results are shown in Table II. The molar ratio of H1 histone/DNA I^r in the P complex had a value of between 40 and 45 over a large range of *R* values. Complexes with less than about 25–30 molecules of H1 histone per DNA I^r were not observed. It should be noted that complete neutralization of the charges on the DNA by H1 histone would occur at an *R* value of 144.

Since no complexes of intermediate sedimentation velocity were observed, the results suggest that the binding of H1 histone to DNA I^r is cooperative. Further support for cooperative interaction comes from preliminary analysis of H1 histone·DNA I^r complexes (fixed according to the method of Christiansen & Griffith, 1977) on metrizamide isopycnic gradients which revealed a bimodal distribution of DNA I^r between free DNA and H1 histone-bound DNA (data not shown). In addition, sigmoidal binding curves were observed when the interaction of H1 histone with DNA I^r was studied by the nitrocellulose filter binding assay (Vogel & Singer, 1975a; and see also Figure 7, below).

H1 Histone·DNA I Complexes. The interaction of H1 histone with supercoiled DNA I gave rise to a type of complex that was not observed with relaxed DNA (Figures 1d–f). At low *R* values nearly all of the H1 histone cosedimented with the DNA I as a slowly sedimenting complex (S complex) at a position indistinguishable on the gradients from that of free superhelical DNA. Only a very small fraction of the H1 histone was recovered as a P complex (Table III). With increasing *R* values at a constant DNA I concentration, the proportion of P complex increased and the proportion of DNA recovered as slowly sedimenting material decreased. However, the S complex persisted at *R* values as high as 45–50. At *R* values higher than about 50 there was virtually no remaining S complex.

The calculated molar ratio of H1 histone to DNA I in the peak of S complex increased with increasing *R* values, but appeared to plateau at a value between 10 and 12 (Table III). It has not been possible to determine if the S complex arises as a result of cooperative or noncooperative interactions of H1 with DNA I. At low *R* values, there was no detectable change in the sedimentation velocity of the S complex relative to that of free DNA. The molar ratio of H1 histone to DNA I in each fraction across an S complex peak was relatively constant (Figure 1d), consistent with the interpretation that the H1 histone is randomly distributed among all DNA molecules in the S complex peak. At higher *R* values, the molar ratio of H1 histone to DNA I in each fraction across an S complex peak increased, consistent with the formation of heavier complexes (Figures 1e and 1f). The maximum molar ratio observed in a fraction of the S complex peak was 16 H1 histone molecules per DNA I molecule. Analysis on isopycnic metrizamide gradients of complexes fixed according to the procedure of Christiansen & Griffith (1977) demonstrated the presence of some free DNA I at low *R* values: the amount of free DNA I decreased with increasing *R* value. However, separation of the various H1 histone DNA I complexes on these gradients was inadequate to distinguish clearly between cooperative and noncooperative mechanisms. Earlier studies, which showed the H1-dependent accumulation of superhelical DNA on nitrocellulose filters to be a linear function of the H1 histone concentration (Vogel & Singer, 1975a; Singer & Singer, 1976; see also Figure 7, below) are compatible with the interpretation that the S complex arises from a noncooperative interaction of H1 histone with superhelical DNA.

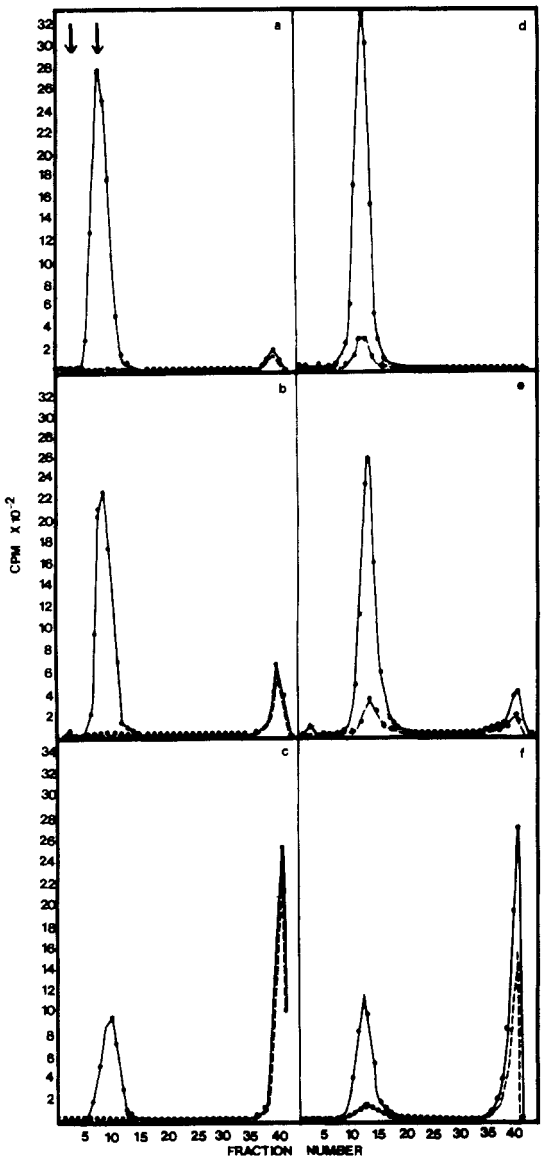

FIGURE 2: Sedimentation velocity gradient profiles of complexes of H1(AB) histone with DNA. [^{32}P]H1(AB) histone·[^{3}H]DNA complexes were formed at different R values and separated on 5–20% metrizamide gradients as detailed in the text. Sedimentation was from left to right. The arrows mark the positions of free [^{32}P]H1(AB) histone and free DNA. (a–c) [^{32}P]H1(AB) histone·DNA I^{r}; (a) R = 8.2; (b) R = 17.8; (c) R = 46.8. Specific activities were: [^{32}P]H1(AB) histone, 5154 cpm/μg; [^{3}H]DNA I, 2310 cpm/μg. (d–f) [^{32}P]H1(AB) histone·DNA I: (d) R = 10.2; (e) R = 19.3; (f) R = 41.8. Specific activities were: [^{32}P]H1(AB) histone, 6357 cpm/μg; [^{3}H]DNA I, 3138 cpm/μg. [^{3}H]DNA (—); [^{32}P]H1(AB) histone (- - -).

As in the case of DNA I^{r}, the P complex formed between H1 histone and DNA I appears to result from a cooperative interaction, since no complexes of sedimentation velocity intermediate between those of S complex and P complex were observed. The molar ratio of H1 histone to DNA I in the P complex increased with increasing R value (Table III). At all R values, the molar ratio of H1 histone to DNA I in the P complex was greater than that of the rapidly sedimenting H1 histone·DNA I^{r} complex formed at the same R value.

Binding of Carboxy-Terminal Fragment 107–212 of H1 Histone to DNA. Earlier studies from this laboratory demonstrated that the globular region of the H1 histone molecule, extending from amino acid residues 73–106, is required for the ability to discriminate between DNA I and DNA I^{r} (Singer & Singer, 1976). A carboxy-terminal fragment of H1 histone

TABLE IV: Distribution of DNA between Slowly and Rapidly Sedimenting Material upon Interaction with Carboxyl-Terminal Fragment 107–212 of H1 Histone.[a]

	Slowly sedimenting DNA (%)		Rapidly sedimenting DNA (%)	
R	DNA I	DNA I^{r}	DNA I	DNA I^{r}
15.7	87.7	89.0	12.3	11.0
31.4	60.0	71.9	40.0	28.1

[a] Complexes of carboxy-terminal fragment 107–212 of H1 histone and [^{14}C]DNA I (sp act. = 4962 cpm/μg) or [^{3}H]DNA I^{r} (sp act. = 21 283 cpm/μg) were formed separately in standard binding buffer and analyzed on gradients, as detailed in the text. The distribution of each form of DNA in slowly and rapidly sedimenting material was calculated; the results are expressed as the percentage of the total DNA recovered.

TABLE V: Molar Ratios of H1(AB) Histone/DNA I^{r} in the Rapidly Sedimenting Complex at Various Input Ratios.

R	H1(AB) in P complex (%)	DNA I^{r} in P complex (%)	H1(AB)/DNA I^{r} (molar ratio)
8.2	76	5	69
17.8	88	14	76
26.7	94	27	73
36.1	95	41	73
46.8	97	59	72
70.4	98	98	75

generated by chymotrypsin cleavage, which extends from amino acid residue 107–212, binds both forms of DNA equally well as assayed by a nitrocellulose filter assay (Singer & Singer, 1976). The ability of this carboxy-terminal fragment to generate P complex with DNA I and DNA I^{r} was examined. Complexes of the carboxy-terminal fragment 107–212 of H1 histone and DNA I or DNA I^{r}, at R values of 15.7 and 31.4, were analyzed on sedimentation velocity gradients. Since in this case only the DNA was radioactively labeled, the fraction of DNA recovered as P complex was compared for the two substrates. The results (Table IV) clearly indicate that, at both of the R values tested, the proportions of DNA I and DNA I^{r} found as P complex were similar. No complexes of intermediate sedimentation velocity were observed in either case. It is not known whether the DNA which sedimented in the position of free DNA in these experiments was present as free DNA or in a complex with fragment. Since the carboxy-terminal fragment 107–212 of the H1 molecule is able to generate P complexes, the globular region of the H1 histone molecule is not necessary for the cooperative binding of H1 histone to DNA.

Phosphorylated H1 Histone·DNA Complexes. To investigate further the basis of the difference in binding of H1 histone to superhelical and relaxed DNA, the effect of phosphorylation of H1 histone at both serine-37 (site A) and serine-106 (site B) on complex formation was examined. Complexes of H1(AB) histone and DNA I^{r}, formed with various amounts of ^{32}P-labeled H1(AB) histone and a constant amount of [^{3}H]DNA, were analyzed on sedimentation velocity gradients. Typical profiles of gradients of these complexes are shown in Figures 2a–c.

At all R values, all of the H1(AB) histone was bound to the DNA I^{r} and, as with nonphosphorylated H1 histone, only one type of complex was formed—a rapidly sedimenting complex (P complex). The amount of this complex formed was pro-

TABLE VI: Molar Ratios of H1(AB)/DNA I in the Slowly and Rapidly Sedimenting Complexes.

	S complex			P complex		
R	H1(AB) in S complex (%)	DNA I in S complex (%)	H1(AB)/DNA I (molar ratio)	H1(AB) in P complex (%)	DNA I in P complex (%)	H1(AB)/DNA I (molar ratio)
4.4	100	100	3.4	0	0	
10.2	100	100	8	0	0	
19.3	62	88	10	37	11	48
31.6	26	62	11	73	37	51
41.8	13	41	10	87	58	50
50.3	5	21	11	94	78	52

portional to the amount of H1(AB) histone present (Table V); a peak of free DNA was also observed which decreased with increasing H1(AB) histone concentration. As observed for nonphosphorylated H1 histone, the binding of H1(AB) histone to DNA I^r appears to be cooperative since no complexes of intermediate sedimentation velocity were observed. From the specific radioactivities of the [^{32}P]histone and [^{3}H]DNA I^r, the molar ratio of H1(AB) histone to DNA I^r in the P complex was calculated (Table V). In the range of R values tested, the molar ratio in the complex ranged from about 70 to 75. This value is almost twice that observed for the nonphosphorylated H1 histone·DNA I^r complex. Therefore, although the binding of H1(AB) histone to DNA I^r is qualitatively the same as the nonphosphorylated histone, there appears to be a quantitative difference.

The nature of the complexes formed by the interaction of H1(AB) histone with superhelical DNA was examined in a similar manner (Figures 2d–f) and again gave results comparable to those described above for nonphosphorylated H1 histone. All of the H1(AB) histone was bound to the DNA at all of the concentrations tested. At low R values, only a slowly sedimenting complex (S complex) was formed; it sedimented at a position indistinguishable from that of free DNA under the conditions used. At higher R values, there was a decrease in the amount of S complex present and a concomitant appearance of a rapidly sedimenting complex (P complex) (Table VI). The molar ratios of H1(AB) histone/DNA I in each of these two complexes are given in Table VI. In the S complex, the maximum number of H1(AB) histone molecules bound per DNA I molecule was 10–11. The molar ratio of H1(AB) histone to DNA I at low R values was constant in each fraction across an S complex peak, compatible with a noncooperative mechanism of binding (data not shown). In the P complex, the calculated molar ratio was between 48 and 50. The binding of the H1(AB) histone to DNA I to form the P complex appears to be cooperative, since no complexes of intermediate sedimentation velocity between the S complex and the P complex were formed. At a given R value, the distribution of the DNA between the two complexes was the same with either H1(AB) or H1 histones.

Interaction with DNA of H1 Histones Phosphorylated at Various Sites. The sedimentation velocity studies described above indicate that at low R values the preferential retention of DNA I (compared with DNA I^r) on nitrocellulose filters is due to the presence of S complex. Since the interaction of H1(AB) histone with DNA I also results in the formation of S complex at low R values, it would be predicted that, in a nitrocellulose filter binding assay, H1(AB) would also display an apparent preferential interaction with superhelical DNA relative to relaxed DNA. The abilities of H1(AB) and three other phosphorylated species of H1 histone [H1(A), H1(B), and H1(GA)] to discriminate between superhelical and relaxed DNA were therefore examined by the filter binding assay.

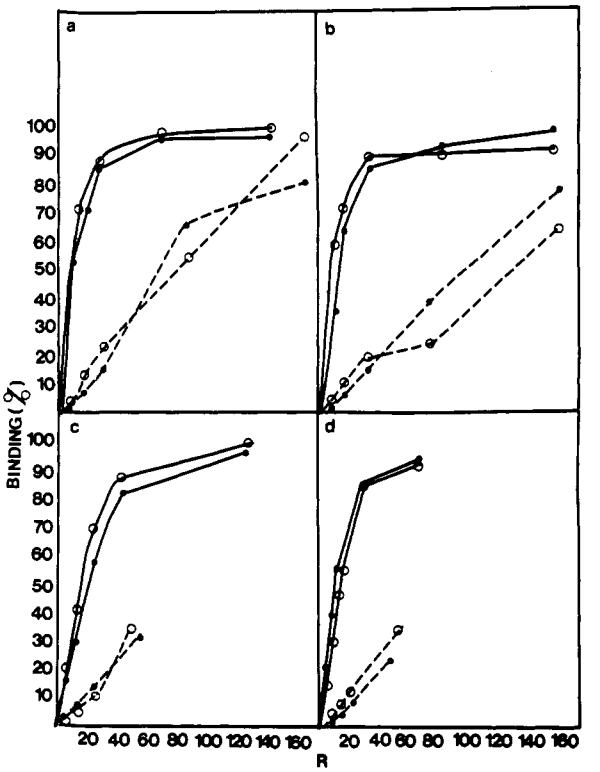

FIGURE 3: Phosphorylated H1 histone-dependent accumulation of [^{14}C]DNA I and [^{14}C]DNA I^r on nitrocellulose filters. (a) H1(A) and control; (b) H1(B) and control; (c) H1(AB) and control; (d) H1(GA) and control. R is the molar ratio of H1/DNA. The specific activity of [^{14}C]-DNA was 11 309 cpm/μg. (●) Phosphorylated H1 histone; (○) nonphosphorylated control; (—) DNA I; (- - -) DNA I^r.

The interactions of each of these species and the corresponding nonphosphorylated controls with DNA I and DNA I^r are shown in Figure 3. There is no detectable difference in binding to DNA between any of these phosphorylated species and the respective controls. There is also no difference between the various controls. Thus, all of the phosphorylated species of H1 histone bind DNA and discriminate between superhelical and relaxed DNA with the same efficiency as the nonphosphorylated controls.

Effect of Salt Concentration on Complex Formation. It was reported recently that the binding of H1 histone to relaxed linear duplex DNA undergoes a transition from noncooperative to cooperative binding as the NaCl concentration is raised from below 20 mM to above 40 mM (Renz & Day, 1976). To investigate further the nature of the interaction of closed circular duplex DNA and H1 histone, the effect of salt concentration on complex formation was examined. H1 histone and DNA

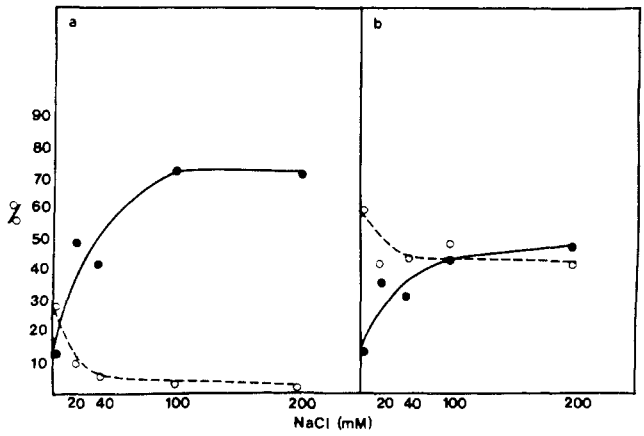

FIGURE 4: Distribution of H1 histone and DNA I^r between slowly and rapidly sedimenting material as a function of the salt concentration. The distributions of H1 histone and DNA I^r between slowly and rapidly sedimenting complexes were calculated following incubation and gradient analysis of the reactants (R = 28.3) at various salt concentrations. The distributions are expressed as the percentage of the total material recovered as either slowly (- - -) or rapidly (—) sedimenting material. At NaCl concentrations below 40 mM, 20–30% of the H1 histone was associated with DNA I^r in complexes of intermediate sedimentation velocity. The distribution of H1 histone and DNA I^r into this component is not shown. (a) H1 histone; (b) DNA I^r.

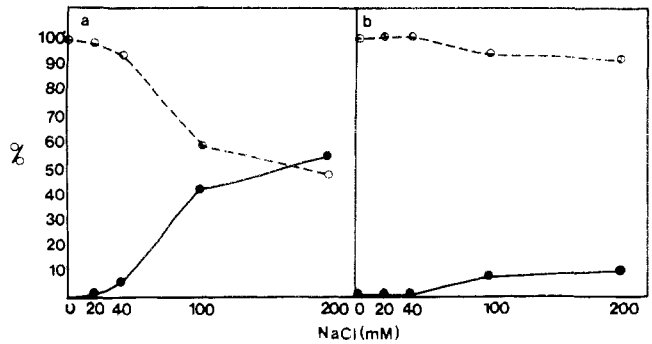

FIGURE 5: Distributions of H1 histone and DNA I between slowly and rapidly sedimenting material as a function of salt concentration. The distributions of H1 histone and DNA I between slowly and rapidly sedimenting material were calculated following incubation and gradient analysis of the reactants (R = 11.8) at various salt concentrations. The distributions are expressed as the percentage of the total material recovered which was recovered as either slowly (- - -) or rapidly (—) sedimenting material. No complexes of intermediate sedimentation velocity were observed at any of the NaCl concentrations. (a) H1 histone; (b) DNA I.

I^r were combined at a constant R value of 28.3 and incubated in binding buffer at concentrations of NaCl ranging from 0 to 200 mM. The resulting complexes were then analyzed on gradients made in the binding buffer at the appropriate salt concentration.

In the absence of any NaCl, only 16% of the H1 histone was found associated with 12% of the DNA I^r in a P complex (Figure 4a). The remaining H1 histone and DNA I^r were associated in the form of either a slowly sedimenting complex or a family of complexes of intermediate sedimentation velocity. Above 20 mM NaCl, the DNA I^r was distributed between P complex and histone-free slowly sedimenting material, whereas nearly all of the H1 histone was associated with DNA I^r in a P complex; no complexes of intermediate sedimentation velocity were observed. Thus above 20 mM NaCl, the results were similar to those for 0.1 M NaCl. This change in distributions of H1 histone and DNA I^r between a slowly sedimenting complex and P complex as a function of salt concentration is illustrated in Figure 4. All of the H1 histone was bound to the DNA I^r at all of the NaCl concentrations tested.

These results therefore confirm those obtained by Renz & Day (1976) with relaxed linear duplex DNA, namely, that the binding of H1 histone to relaxed DNA (in this case a closed circular molecule) undergoes a transition from a noncooperative interaction at low salt to a cooperative interaction at salt concentrations above 20 mM.

The effect of salt concentration on complexes of H1 histone and supercoiled DNA was also examined (Figure 5). At concentrations of NaCl up to 40 mM, little or no P complex was formed (at an R value of 11.8); above 40 mM NaCl, P complex was observed. A marked increase in the proportion of H1 histone found in P complex occurred between 40 and 100 mM NaCl (Figure 5a). An increase in the proportion of DNA in the P complex was also observed over this concentration range (Figure 5b). At all of the NaCl concentrations tested, all of the H1 histone was bound to DNA.

Whereas the largest change in the distribution of H1 histone·DNA I^r complexes occurred between 20 and 40 mM NaCl, the largest change in the distribution of H1 histone·DNA I complexes occurred between 40 and 100 mM. In distinction to the results with DNA I^r at low salt, no H1 histone·DNA I complexes of intermediate sedimentation velocity were observed at any salt concentration.

Effect of NaCl Concentration on the Interaction of Phosphorylated H1 Histones with DNA. The binding of DNA I by calf thymus H1 histone is optimal at 100 mM NaCl, whereas the binding of DNA I^r by H1 histone decreases with increasing salt concentrations, as assayed by the filter binding assay at a constant R value (Vogel & Singer, 1976; Singer & Singer, 1976). The salt optima for the binding of DNA I by the various phosphorylated species of H1 histone were examined. [^{14}C]-DNA I and each of the four phosphorylated H1 histone samples were mixed at a constant R value in binding buffer adjusted to NaCl concentrations ranging from 0 to 200 mM; the extent of complex formation was measured by the filter binding assay. The results are shown in Figure 6. Two of the samples—H1(A) and H1(GA) histones—show the same optimal NaCl concentration for binding of DNA I as the controls, namely, between 50 and 100 mM NaCl. However, the optimal NaCl concentration for binding of DNA I by H1(B) and H1(AB) histones is shifted to a lower salt concentration, namely, between 0 and 20 mM NaCl. In the absence of NaCl, at a given R value, both the H1(B) and the H1(AB) histones bind DNA I better than the nonphosphorylated controls. To investigate this further, the abilities of H1(A), H1(B), and H1(AB) histones to complex DNA were titrated in the absence of NaCl. As shown in Figure 7, at low R values in the absence of NaCl, the nonphosphorylated control H1 histone binds DNA I poorly, although somewhat better than DNA I^r. (At the low H1/DNA ratios tested, little binding of DNA I^r is expected.) There is no marked difference in the binding of DNA I^r by the phosphorylated histones relative to the control. On the other hand, all three phosphorylated H1 histone species bind DNA I much better than the control in the absence of salt. H1(B) and H1(AB) histones are more efficient in binding DNA I than is H1(A) histone under these conditions, although all three are approximately equivalent in their ability to complex DNA I^r. Therefore, phosphorylation of H1 histone at serine-106, which is in the globular region of the molecule, markedly affects the salt dependence of complex formation. Phosphorylation at serine-37 also affects the binding at low salt, but to a lesser degree.

It has been reported that H1 histone·DNA complexes are not bound to nitrocellulose filters in the absence of NaCl (Renz

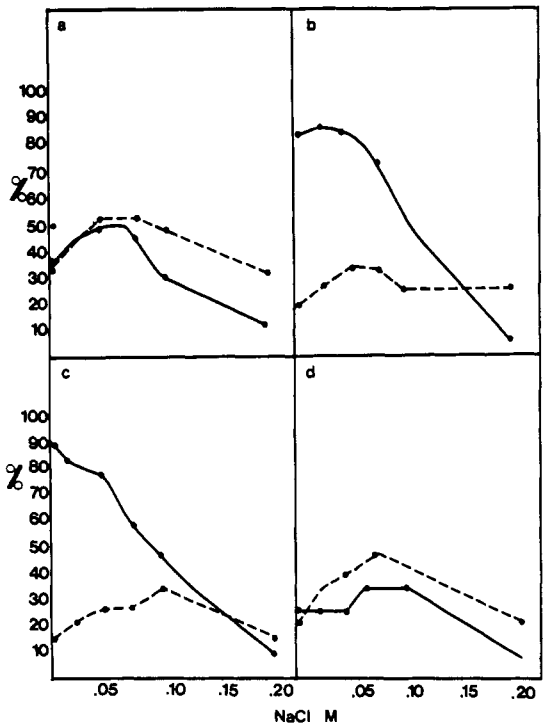

FIGURE 6: The effect of NaCl concentration on the phosphorylated H1 histone dependent accumulation of [^{14}C]DNA I on nitrocellulose filters. The various phosphorylated H1 histone species and controls, at constant R values, were incubated with [^{14}C]DNA I (sp act. 11 309 cpm/μg) in 50 mM Tris, pH 7.8, 1 mg/mL BSA, 1 mM EDTA, and various NaCl concentrations. The results are plotted as the percent of maximum binding. (a) H1(A) and control, R = 9.57; (b) H1(B) and control, R = 9.57; (c) H1(AB) and control, R = 8.52; (d) H1(GA) and control, R = 9.31. (—) Phosphorylated H1; (- - -) nonphosphorylated control.

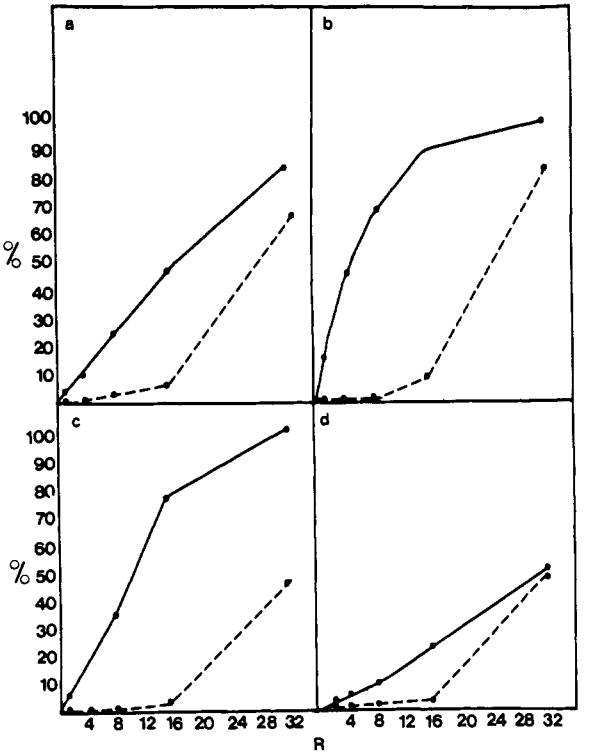

FIGURE 7: Phosphorylated H1 histone-dependent accumulation of DNA on nitrocellulose filters in the absence of NaCl. A constant amount of [^{14}C]DNA (sp act. 11 309 cpm/μg) was incubated with varying amounts of phosphorylated H1 histone species and controls in binding buffer in the absence of any NaCl. The results are plotted as the percent of the input DNA bound to the filter. (a) H1(A); (b) H1(B); (c) H1(AB); (d) H1 control. DNA I (—); DNA I^r (- - -).

& Day, 1976). One possible interpretation of the results presented here is that nonphosphorylated H1 histone·DNA complexes are not bound to the filters in the absence of salt, whereas phosphorylated H1 histone·DNA complexes are bound. The ability of [^{3}H]H1 histone to bind to nitrocellulose filters at various salt concentrations was tested by filtering either [^{3}H]H1 histone alone in binding buffer or as a complex with either DNA I or DNA I^r. The results shown in Table VII clearly demonstrate that [^{3}H]H1 histone in a complex with DNA binds to nitrocellulose filters equally at all salt concentrations tested. The binding to filters of [^{3}H]H1 histone alone decreases slightly with increasing salt. DNA alone does not bind to the filters.

TABLE VII: Relative Binding of [^{3}H]H1 Histone to Nitrocellulose Filters as a Function of Salt Concentration.

[NaCl]	[^{3}H]H1 alone[a]	[^{3}H]H1·DNA I[a]	[^{3}H]H1·DNA I^r[a]
0	100.0 (66.6)	92.5	95.9
20	95.3	99.6	
40	89.3	100.0 (89.6)	100.0 (66.4)
100	75.8	92.4	93.5
200	77.6	89.0	92.0

[a] The binding of [^{3}H]H1 histone to nitrocellulose filters at a given salt concentration is expressed relative to the salt concentration at which binding was maximum. The actual percent of the total input [^{3}H]H1 histone retained on the filter, either alone or as a complex with DNA I or DNA I^r in binding buffer, is given in parentheses.

Discussion

The nature of the binding of DNA by H1 histone is dependent on the tertiary structure of the DNA. Superhelical SV40 DNA contains between 24 and 26 superhelical turns (Keller, 1975; Shure & Vinograd, 1976). Relaxed, closed circular DNA has an average superhelical density of zero. H1 histone binds relaxed, closed circular SV40 DNA in a highly cooperative fashion at 100 mM NaCl, yielding only rapidly sedimenting complexes containing an average of 40–45 molecules of H1 histone per molecule of DNA I^r. However, under the same conditions, the interaction of H1 histone with superhelical SV40 DNA gives rise to two types of complexes: a slowly sedimenting complex and a rapidly sedimenting complex. The relative amount of each complex depends on the R value. The slowly sedimenting complex (S complex) contains an average of 10–12 H1 histone molecules per DNA I molecule. The rapidly sedimenting complex (P complex) arises as a result of a cooperative binding of H1 histone to DNA I: the number of H1 histone molecules per DNA I molecule in the P complex varies as a function of the R value, ranging from 45 to 80.

The present data indicate that the presence of superhelical turns in a closed circular DNA molecule allows a distinct type of interaction: the formation of the S complex at 100 mM NaCl is unique to the interaction of H1 histones with superhelical DNA. The nature of this interaction is still unclear, although the data are suggestive of a noncooperative mechanism. We have previously reported that H1 histone complexes superhelical DNA more efficiently than relaxed closed circular DNA as measured by the H1 histone dependent retention of the H1 histone·DNA complex on nitrocellulose filters (Vogel & Singer, 1975a,b). This apparent preferential binding of H1 histone to superhelical DNA relative to relaxed DNA is now understood to result from the formation of different types of complexes at low R values. Other workers (Böttger et al., 1976)

studying H1 histone·DNA I complexes at R values much greater than those reported here would not have detected the S complex.

The binding of H1 histone to sheared, linear duplex DNA undergoes a transition from noncooperative to cooperative between salt concentrations of 20 and 40 mM (Renz & Day, 1976). The P complex also appears to arise from a cooperative, salt-sensitive interaction. At salt concentrations below 20 mM NaCl, little P complex is formed upon interaction of H1 histone with relaxed, closed circular DNA I^r, although all of the H1 histone is bound to the DNA. P complex is observed at salt concentrations of 20 mM and higher. A similar transition is observed for the H1 histone·DNA I P complex, but occurs at a somewhat higher salt concentration, between 40 and 100 mM.

The salt dependent transition from noncooperative to cooperative binding of DNA by H1 histone could possibly arise from a conformational change in the structure of the H1 histone molecule over this salt range. Salt-induced changes in the NMR spectrum of H1 histone have been reported (Bradbury et al., 1975). Alternatively, it is possible that H1 histone cannot overcome the low salt-induced polyelectrolyte expansion (Felsenfeld & Miles, 1967) of the DNA in order to form P complex. The observation that the transition for superhelical DNA occurs at a higher salt concentration than relaxed DNA would appear to be consistent with this view, since superhelical DNA might be imagined to contain regions of high charge density at helix cross-over points.

Neither the mechanism of the cooperative binding of H1 histone to DNA which results in the P complex nor the structure of the P complex is known. However, the carboxy-terminal portion of the H1 histone molecule is sufficient to achieve cooperative binding. Thus, the interaction of the carboxy-terminal fragment 107–212 of H1 histone with either form of DNA yields a P complex. The presence of the globular region of the H1 histone (amino acid residues 73–106), which has been previously demonstrated to be involved in the recognition of superhelical DNA (Singer & Singer, 1976), does not appear to be necessary for the cooperative binding of H1 histone to either form of DNA. It has been suggested that superhelicity of DNA restricts the cooperative interaction of H1 histone with DNA (Renz & Day, 1976). This does not appear to be the case, since P complexes of DNA I are formed with an efficiency similar to P complexes of DNA I^r.

Phosphorylation of H1 histone does not qualitatively affect its binding to DNA or recognition of superhelical DNA in 100 mM NaCl. H1(AB) histone, like H1 histone, binds DNA I at low R values to give rise to a slowly sedimenting complex containing about 10 molecules of H1(AB) histone per molecule of DNA. At higher R values in 100 mM NaCl, a rapidly sedimenting P complex forms cooperatively and has a molar ratio of 48–52. This molar ratio is somewhat lower than that observed for the P complex of H1 histone·DNA I.

In 100 mM NaCl, H1(AB) histone binds to DNA I^r in a cooperative fashion, yielding a rapidly sedimenting P complex with a molar ratio of 70–75. This molar ratio is markedly different from that of 40–45 obtained with the nonphosphorylated H1 histone under the same conditions. Therefore, the binding of H1(AB) histone to DNA I^r is quantitatively, but not qualitatively, different from nonphosphorylated H1 histone. These results are consistent with those obtained by Adler et al. (1972) who observed that more H1(AB) histone was required than nonphosphorylated control to induce changes in the CD spectrum of linear DNA. Willmitzer et al. (1977) have reported similar differences between phosphorylated and nonphosphorylated protamines.

One qualitative difference in the binding of phosphorylated and nonphosphorylated H1 histone to DNA was differential sensitivity to NaCl concentration. H1(AB), H1(B), and, to a lesser extent, H1(A) histone bound and recognized superhelical DNA, in the absence of NaCl, more efficiently than the nonphosphorylated controls. This difference was not due to a differential retention of the histone·DNA complexes by the nitrocellulose filters at the various salt concentrations. These data suggest that modification of the H1 histone by phosphorylation in the globular core of the molecule stabilizes its ability to bind superhelical DNA in the absence of salt.

Although the H1 histone content of chromatin is not known with certainty (Johns, 1967; Panyim & Chalkley, 1969; Oliver & Chalkley, 1972; Fambrough et al., 1968; Olins et al., 1976), the results presented here seem to be of particular interest in relation to the current concepts of chromatin structure. If the DNA in the nucleosomes is recognized by the H1 histone as supercoiled, the present studies would predict that the interaction of H1 histone with chromatin could lead to two types of structures: an extended structure corresponding to the S complex and a compacted structure corresponding to the P complex. Conversion between the two forms might be brought about by local changes in H1 histone concentration. Preliminary studies indicate that the binding of H1 histone to SV40 minichromosomes prepared from virions gives rise to an S complex at low R values (Singer & Singer, unpublished observations). Indeed, it has been reported recently that the addition of H1 histone to SV40 minichromosomes, at an R value of approximately 25, results in a marked condensation of the minichromosome structure (Bellard et al., 1976). Cellular euchromatin, which is an extended, transcriptionally active structure, is relatively depleted in H1 histone, whereas heterochromatin, which is a highly condensed, transcriptionally inactive structure, is relatively enriched in H1 histone (Elgin & Weintraub, 1975).

It is evident from the results presented here that the phosphorylation of H1 histone has a greater effect on the binding to relaxed DNA than on supercoiled DNA. In chromatin, regions of relaxed DNA (internucleosomal DNA) alternate with regions of supercoiled DNA (nucleosomal DNA). Noll (1976) has proposed that the length of internucleosomal DNA is determined in part by the association of H1 histone with chromatin. Recent evidence suggests that, during the development of the sea urchin from sperm to the gastrula stage, there are changes in both the pattern of H1 histone synthesis (Arceci et al., 1976) and in the length of internucleosomal DNA (Spadafora et al., 1976). It is possible to speculate that, in the chromatin of mature cells, the phosphorylation of H1 histone may mediate changes in the length of internucleosomal DNA. Since phosphorylation of H1 histone does not qualitatively alter its binding to DNA, it would be possible to achieve changes in the degree of higher order packaging of nucleosomes without causing major alterations in the nature of the H1 histone binding to a superhelical substrate.

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