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Cooperative binding of the globular domains of histones H1 and H5 to DNA

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ABSTRACT

In view of the likely role of H1-H1 interactions in the stabilization of chromatin higher order structure, we have asked whether interactions can occur between the globular domains of the histone molecules. We have studied the properties of the isolated globular domains of H1 and the variant H5 (GH1 and GH5) and we have shown (by sedimentation analysis, electron microscopy, chemical cross-linking and nucleoprotein gel electrophoresis) that although GH1 shows no, and GH5 little if any, tendency to self-associate in dilute solution, they bind highly cooperatively to DNA. The resulting complexes appear to contain essentially continuous arrays of globular domains bridging 'tramlines' of DNA, similar to those formed with intact H1, presumably reflecting the ability of the globular domain to bind more than one DNA segment, as it is likely to do in the nucleosome. Additional (thicker) complexes are also formed with GH5, probably resulting from association of the primary complexes, possibly with binding of additional GH5. The highly cooperative nature of the binding, in close apposition, of GH1 and GH5 to DNA is fully compatible with the involvement of interactions between the globular domains of H1 and its variants in chromatin folding.

INTRODUCTION

The importance of histone H1 and its variants in mediating the transition *in vitro* between the extended 10 nm nucleosome filament and the folded 30 nm filament is well established (1–3, and references therein). Chemical cross-linking (4) suggests a polar, head-to-tail (N- to C-) array of H1 molecules in close juxtaposition, possibly in contact, along the nucleosome filament and, additionally, closely juxtaposed C-terminal domains in the folded state (5). If H1-H1 interactions exist in the 30 nm filament (1) and are cooperative, as they are generally assumed to be [e.g. (6)], this would provide a means *in vivo* of stabilizing or destabilizing whole domains of chromatin structure, for example in transcriptionally inert and competent states respectively. In transcriptionally repressed nuclei, such as those from chicken erythrocytes and sea urchin sperm, distinctive H1 variants (H5 and spH1 respectively) replace, largely or completely, the 'normal' H1, and may contribute significantly (in concert with lengthening of the nucleosome repeat length) to the enhanced stability of chromatin higher order structures from these sources, relative to rat liver chromatin (7, and references therein).

H1 shows no tendency to self-associate in dilute solution under conditions in which H1 polymers are formed by cross-linking in chromatin (4), but at ionic strengths above ~30 mM (and lower for H5 and spH1) all the variants bind cooperatively to DNA (8, 9). The cooperatively formed complexes at this ionic strength consist of intertwined 'thin filaments' which appear from electron microscopy to consist of 'tramlines' of two DNA duplexes bridged by an array of closely apposed H1 (or variant) molecules; the thickness of the complexes suggests that the separation of the DNA molecules corresponds roughly to the diameter of the histone globular domain [~29 Å (10, 11)]. The spacing determined for H1 molecules along the DNA is larger than would allow direct interactions between globular domains in these complexes, and may well reflect the disposition of H1 molecules along the nucleosome filament (9). However, the folding of the nucleosome filament into the chromatin higher order structure could well be the result of interactions between the globular domains of H1. We have therefore asked whether there is any evidence of homomeric interactions between isolated globular domains of H1 or H5 (GH1 and GH5 respectively; ~80 amino acid residues), either free in solution or bound to DNA, and if so whether there are differences between GH1 and GH5 that might reflect interactions of different strength in chromatin higher-order structures of different stability (12).

The results demonstrate cooperative binding of GH1 and GH5 to DNA, and an inherent ability to self-associate in the presence of DNA that would be consistent with a role for interactions between globular domains of the H1 histones as a driving force, or stabilizing feature, in chromatin folding.

MATERIALS AND METHODS

Isolation and characterisation of the globular domains of H1 and H5

Chicken erythrocyte nuclei were isolated as described (13). H1 and H5 were extracted with 5% (w/v) perchloric acid by swirling intermittently on ice for 20 min; after centrifugation the extract was immediately neutralized with undiluted triethanolamine,

dialysed against 10 mM Na phosphate, pH 7.0, 0.1mM phenylmethylsulphonyl fluoride (PMSF), and H1 and H5 were separated by chromatography on carboxymethyl cellulose (Whatman CM-52) (8).

Excision of the globular domains, GH1 and GH5, by removal of the N- and C-terminal tails was carried out by tryptic digestion of H1 and H5 in 0.5 M NaCl, 10 mM Na phosphate, pH 7 at 23°C, based on an analytical time course of digestion [typically for 20 min; H1 or H5 at 1 mg/ml; 1:50 (w/w) trypsin:histone], to give a metastable end-product of digestion migrating just ahead of H4 in an SDS/18%-polyacrylamide gel (14). Digestion was stopped by chilling at 4°C and addition of PMSF to 2 mM (from a 50 mM stock solution in propan-2-ol). Agarose-linked soybean trypsin inhibitor (Sigma) was added (15 μl packed gel per μg trypsin: a 3-fold excess) and the suspensions were dialysed overnight against 10 mM Na phosphate pH 7, 0.5 mM PMSF. The agarose beads were removed by centrifugation and GH1 and GH5 were purified by chromatography on carboxymethyl cellulose (Whatman CM52) using a linear gradient of 0.3−0.8 M NaCl in 10 mM Na phosphate, pH 7, 0.5 mM PMSF; the eluate was monitored at 230 nm and by SDS/18%−polyacrylamide gel electrophoresis. GH1 (which eluted from CM 52 at ~0.3 M NaCl) and GH5 (elution at ~0.4 M NaCl) were dialysed against 0.1% (w/v) ammonium hydrogen carbonate and freeze-dried. When required, solutions were prepared in 1mM Na phosphate, pH 7, and the protein concentrations were determined by amino acid analysis (8, 9) or from the A_{230} (A_{230} = 4.5 in a 1 cm pathlength for 1 mg/ml GH5, and 2.8 for 1 mg/ml GH1, determined by amino acid analysis).

N-Terminal sequence analysis was carried out for 10 cycles on ~250 pmol GH1 or GH5 using an Applied Biosystems 470A sequencer coupled to a 120A on-line PTH-analyser. Molecular masses of GH1 and GH5 were determined on similar amounts by electrospray mass spectrometry (15) carried out by Mr B.N.Green (VG BioTech, Altrichnam, U.K.) using a VG BioQ quadrupole mass spectrometer fitted with an electrospray ionization source as described (16), except that the mass scale was calibrated with ubiquitin (mass 8564.9 Da).

Formation and sedimentation of globular domain-DNA complexes

The DNA used for complex formation with GH1 and GH5 was ~146 bp DNA from chicken erythrocyte nucleosome core particles (17); or ~200, 400, 600 or 800 bp DNA extracted from mono-, di-, tri- or tetranucleosomes from a micrococcal nuclease digest size-fractionated in sucrose gradients (8); or 'long DNA' (~4000−9000 bp, weight average size ~6000 bp) extracted from partially fractionated long nucleosome oligomers.

Mixtures of GH1 or GH5 and DNA of various sizes were prepared in 5 mM triethanolamine (TEA)/HCl, pH 7.5, 5 mM NaCl and incubated for 30 min at 24°C essentially as described for H1-DNA and H5-DNA complexes (9), the DNA being added last to a final concentration of 50 μg/ml.

The reaction mixtures (~0.5 ml) containing 25 μg of 'DNA were loaded directly on to 12 ml 5−30% (w/v) linear sucrose gradients containing 5mM TEA/HCl, 5 mM NaCl, pH 7.5, and centrifuged for 16−24 h at 29 000−40 000 rev/min, as indicated, in a Beckman SW40 rotor. Gradients were fractionated and monitored at 280 nm (18), and fractions were analysed for protein content in SDS/18%-polyacrylamide gels (14). Distinct peaks of complexes, sedimenting faster than DNA, were analysed by electron microscopy and subjected to acid hydrolysis for amino acid analysis [c.f. 8, 9)].

Nucleoprotein gel electrophoresis

Mixtures of GH1 or GH5 and DNA were prepared as described above and 0.6 A_{260} unit was loaded on to a 5% polyacrylamide gel containing 10 mM Tris/HCl, pH 7.5, 1 mM Na_2EDTA (19) which been pre-run at 100V for 1h in the cold room, with a buffer change after ~30 min. Samples fractionated in sucrose gradients were loaded directly in sucrose. Electrophoresis was carried out using the same buffer in the cold room at 100V (with a buffer change after 45 min) for about 1.5 h, until the bromophenol blue marker (loaded into empty tracks) was 1 cm from the bottom of the gel. The gel was stained with ethidium bromide and photographed with transillumination with short wavelength ultraviolet light.

Electron microscopy

Mixtures of GH1 and GH5 with DNA, or complexes isolated from sucrose gradients, were fixed for ~18 h at 4°C with 0.1% (v/v) glutaraldehyde and examined by electron microscopy after rotary shadowing with Pt (1). The samples that were fixed in sucrose were dialysed into 5 mM TEA/HCl, 5 mM NaCl, pH 7.5, before electron microscopy.

Chemical cross-linking

GH1-DNA or GH5-DNA complexes, unfractionated or taken from sucrose gradients, were treated at 23°C with 0.3 mg/ml dithiobis(succinimidyl propionate) (DSP; from Pierce; stock solution 30 mg/ml in dimethylformamide) for up to 20 min, as indicated, based on a time-course (20). The cross-linked products were analysed in SDS/18%-polyacrylamide slab gels which were stained with Coomassie Blue R250 and/or silver-stained (21).

Protein-DNA composition of complexes

Compositions were determined for fractions from sucrose gradients, taken from the middle of the well-defined peaks of A_{280}-absorbing material containing GH5- or GH1-DNA complexes. Samples from the gradients were dialysed against 5mM TEA/HCl, 5 mM NaCl, pH 7.5; the DNA content was determined from the A_{260} (taking A_{260} = 20 as 1mg DNA/ml, in a 1 cm pathlength); the protein content was determined by amino acid analysis in duplicate after hydrolysis of ~0.02−0.1 A_{260} units of complex, as described previously for H1(H5)/DNA complexes (8, 9).

RESULTS

Isolation and characterisation of GH1 and GH5

GH1 and GH5 were generated from H1 and H5 by tryptic digestion in the presence of 0.5−0.7 M NaCl (Figure 1), which stabilizes the folded domains [estimated as residues 35−120 in GH1 (22) and 22−100 in GH5 (10)], and purified by chromatography on carboxymethyl cellulose (elution at ~0.3 M and ~0.4 M NaCl respectively, in a salt gradient); the products were homogeneous as judged by SDS-gel electrophoresis (e.g. see Figure 8). N-Terminal sequence analysis of GH5 indicated a major sequence ^{22}S-A-S-H-P-T-Y-S-E-M-.... [numbering based on the H5 sequence (23)] and a minor one with an additional arginine residue at the N-terminus. Electrospray mass spectrometry (not shown) showed that GH5 contained three significant components ; the molecular masses, taken in

conjunction with the N-terminal sequence analysis, defined the C-terminal cleavage points. The major component (molecular mass 8712.6 Da) was thus Ser 22−Lys 102 (81 residues; mass calculated from the amino acid sequence, 8712.0 Da); the two minor components (masses 8869.1 Da and 8513.4 Da) were Arg 21−Lys 102 and Ser 22−Lys 100 (calculated masses 8868.2 Da and 8512.8 Da, respectively).

Sequence analysis of GH1 was also consistent with two related N-terminal sequences, (^{32}A-R-)K-P-A-G-...., differing by A-R- as shown; amino acid analysis was consistent with C-terminal cleavages around residues 115−120 [numbering as in (24)]. Electrospray mass spectrometry revealed a mixture of related species which was more complex than for GH5, due both to heterogenous C-terminal (as well as N-terminal) cleavages, and to the existence of six closely related sequence variants of H1 (25). Analysis of the spectrum (together with the sequence information) indicated that C-terminal cleavages occurred mainly at Lys 120 and Lys 115, with minor cleavages at Lys 117 and Lys 121. The major component was Ala 32−Lys 120 (89 residues). [The numbering is for cleavages in the sequence for one of the H1 variants (24); cleavages occurred at corresponding positions (26) in the other variants.] GH1 as isolated from bulk chicken erythrocyte H1 after tryptic digestion is thus a mixture of closely related species with 'ragged ends', containing a total of 84−89 amino acid residues, and slightly larger than GH5 (81−82 residues).

GH1 and GH5 bind cooperatively to DNA

Mixtures of GH1 and DNA at various protein:DNA ratios from 10−60% (w/w) at low ionic strength (5 mM triethanolamine/HCl, pH 7.5, 5 mM NaCl) were analysed by sedimentation through sucrose gradients. Figure 2 shows the results for ~600 bp DNA. These input weight ratios represent on average ~5−30 molecules of GH1 added per DNA fragment; in principle a DNA fragment ~600×3.4 Å long would be able to accommodate ~70 linearly juxtaposed globular domains, each ~29 Å in diameter (11). Even at low protein:DNA ratios a more rapidly sedimenting population of DNA fragments is evident, which increases with increasing GH1 input ratio (arrowheads). From SDS-gel electrophoresis (not shown) this was found to be associated with all the input GH1; the slower sedimenting material is naked DNA. This concentration of protein on a subpopulation of the DNA fragments is clear evidence of cooperative binding. Similar results were obtained for GH1 complexes with DNA of ~400 and 800 bp (not shown). Essentially all of the input DNA was recovered in the two peaks within the gradient up to an input ratio of ~40−50%; beyond this there was a significant recruitment of DNA into a pellet. This is evident from the gradient profile for an input ratio of 60% in Figure 2 (which also shows a rapidly sedimenting aggregate that has not quite reached the bottom).

The cooperative nature of the binding of GH1 to DNA was also apparent from electron micrographs. Figure 3 shows the co-existence of free ~800 bp DNA and complexes of roughly the same length with diameter ~75 Å (after Pt-shadowing) even at

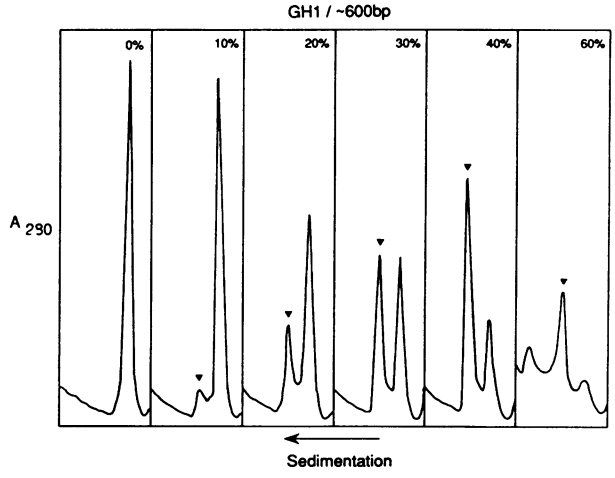

Figure 2. GH1 binds cooperatively to ~600 bp DNA as judged by sedimentation analysis. Mixtures (0.5 ml) containing 25 μg of ~600 bp DNA and increasing ratios of GH1 to DNA (10−60%, w/w) in 5 mM TEA/HCl, pH 7.5, 5 mM NaCl were analysed in 5−30% (w/v) sucrose gradients centrifuged in a Beckman SW40 rotor at 30 000 rev./min for 16.5 h. DNA was located from the A_{280}, and GH1 by precipitation of fractions with 25% (w/v) trichloroacetic acid and analysis in SDS/18%-polyacrylamide gels. Arrowhead indicates the growing peak of complex sedimenting ahead of the DNA and containing essentially all the detectable protein in the gradient.

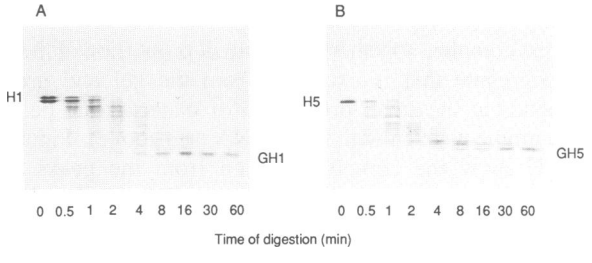

Figure 1. Generation of GH1 (**A**) and GH5 (**B**) by tryptic digestion of H1 and H5 at high ionic strength. H1 or H5 at 2 mg/ml in 0.7 M NaCl, 10 mM Na phosphate, pH 7, were digested with trypsin (1:100 (w/w) enzyme : protein) at 23°C and aliquots were analysed after various times by SDS/18%-polyacrylamide gel electrophoresis. (Identical results were obtained with 0.5 M NaCl.) Gels were stained with Coomassie Blue.

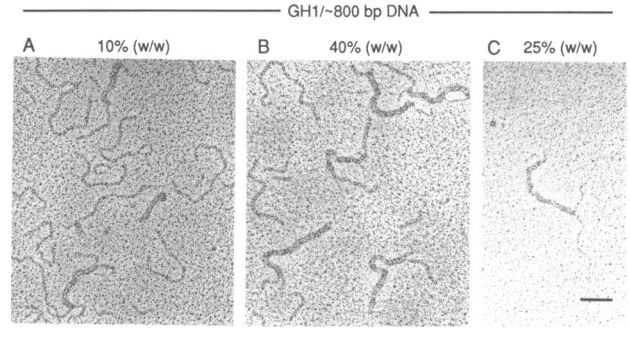

Figure 3. Electron microscopy of GH1/~800 bp DNA mixtures reveals cooperative binding. **A** and **B**. Mixtures at input weight ratios of 10% and 40%. Incubation mixtures containing 1.25 μg DNA complexed with 10% or 40% GH1 in a final volume of 25 μl were diluted to 75 μl with incubation buffer and fixed with 0.1% glutaraldehyde before electron microscopy (1). **C**. A single (but not atypical) complex from a similar mixture at 25% (w/w) input, selected to show the two DNA strands at one end, presumably resulting from incomplete coating by protein. Scale bar represents 50 nm. (The slightly thicker appearance of the free DNA molecules in A and B than in C and other Figures is rather atypical, and is due to the shadowing of that set of grids.)

a GH1:DNA input ratio as low as 10% (~6.5 mol GH1/mol DNA); at the higher input ratio (40%, w/w) the proportion of complexes was higher. Two DNA molecules protrude from the ends of some of the complexes (Figure 3C), consistent with the presence of two duplexes (each ~20 Å across) bridged by a closely packed array of GH1 molecules (diameter ~29 Å).

The behaviour of GH5 with long DNA (e.g. ~800 bp ; not shown) was broadly similar to that of GH1, in that free DNA coexisted with complexed DNA, indicating cooperative behaviour. However, there were several differences: firstly, a broader complex peak in the gradient than with GH1, indicating greater heterogeneity; secondly, increasing loss of DNA into material that sedimented to the bottom of the sucrose gradient under these conditions as the protein:DNA ratio was increased above 20–30% (w/w), which was similar for ~400– 800 bp DNA; and, thirdly, recruitment of a small proportion (a few percent) of the DNA with a substantial proportion of the protein (~20–50%), at essentially all input ratios, into a very rapidly sedimenting aggregate that could be removed by centrifugation for 10 minutes in a microfuge.

Differences between GH1 and GH5 complexes with long DNA were to some extent also apparent from electron microscopy of unfractionated mixtures (Figure 4). Some complexes ~75 Å thick, similar to the GH1 complexes, were evident, particularly at input ratios up to ~20% (w/w) [Figure 4B (a)], but at higher input ratios an increasing proportion of the complexes was thicker (~150 Å) [Figure 4B (b)], and these presumably correspond to the rapidly sedimenting aggregate lost to the bottom of the sucrose gradient with increasing protein:DNA input ratio. They resembled, at least superficially, the intramolecularly self-associated regions of complexes formed with long DNA (weight average ~6000 bp) and GH1 (Figure 4C), suggesting that the thicker GH5 complexes might correspond to doubled-up versions of the thinner complexes (possibly intertwined, since the thicker complexes were mostly somewhat shorter than the thinner complexes for a given DNA size). Further insights came from studies with short DNA described below.

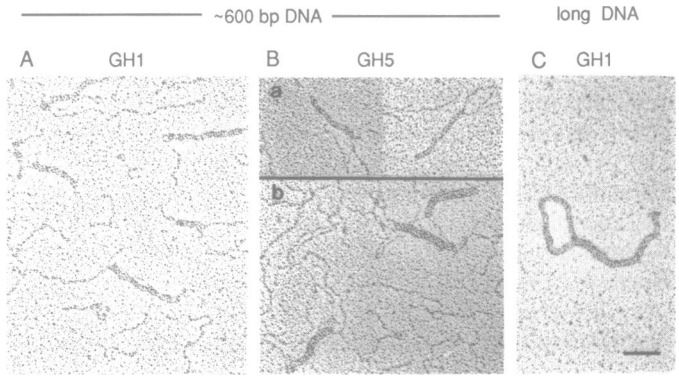

Figure 4. Comparison by electron microscopy of complexes of GH1 and GH5 with DNA. **A** and **B**. Unfractionated mixtures of GH1 and GH5, respectively, with ~600 bp DNA at 40% w/w input (~20 mol G/mol DNA) [A and B (b)], or 20% [B (a)]. **C**. A single complex of GH1 and long DNA at the same input ratio [the DNA had a weight-average size of ~6000 bp with a broad range (4000–9000 bp)]; note the uniform coating and the thicker regions arising from intertwining of thinner regions. Complexes were prepared and examined exactly as for Figure 3. Scale bar represents 50 nm.

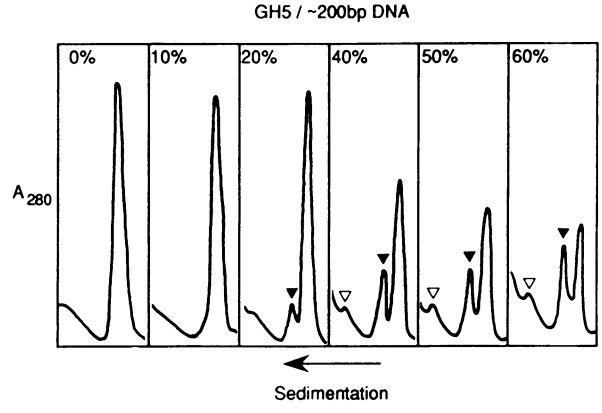

Figure 5. Two classes of complex, rapidly and more slowly-sedimenting, are formed by cooperative binding of GH5 to ~200 bp DNA. Complexes were formed at various input ratios (10–60%) of GH5 to ~200 bp DNA (~1.7–10 mol GH5/mol DNA) as described (Materials and Methods), except that the DNA concentration was reduced to 25 mg/ml; 0.8 ml samples were applied to 5–30% (w/v) sucrose gradients which were centrifuged in a Beckman SW40 rotor at 29 000 rev./min for 16 h. Solid arrowhead, slow-sedimenting complex; open arrowhead, fast-sedimenting complex apparent at input ratios above ~20%.

Complexes with shorter DNA

The behaviour of GH1 and GH5 in complex formation with shorter DNA (~200 bp from mononucleosomes, or ~146 bp from nucleosome core particles) was examined because it offered the possibility of analysis in nucleoprotein gels as well as by sedimentation and electron microscopy, and it also seemed possible that, in the case of GH5, losses by aggregation might be less than with longer DNA.

Well resolved gradient profiles were obtained for GH5 with ~200 bp DNA (Figure 5), although the proportion of the input DNA found in complexes sedimenting within the gradient was still rather less than for GH1 (not shown), suggesting that there was still some loss by aggregation. The most noticeable difference between GH5 and GH1, however, was the appearance with GH5 of a second, small, rapidly sedimenting, broad peak of protein-DNA complexes, that was contained within the gradient for this short DNA (see below).

Confirmation of differences between GH1 and GH5 complexes came from nucleoprotein gel electrophoresis. Figure 6A shows mixtures containing ~146 bp DNA and 25% (w/w) GH1 and GH5. Both contain a major complex migrating about half as fast as free DNA, the GH5 complex having a slightly higher mobility [close to that of nucleosome core particles (c.p.)]. In addition, GH5 gives rise to a second complex migrating about half as fast as the first complex, together with a small proportion of the DNA in an aggregate that is excluded from the gel and probably corresponds to the small proportion of material that pellets in sucrose gradients even with short DNA (see above). Figure 6B, C, D, E show the complexes taken from the peaks in the corresponding sucrose gradients (Figure 6B′, C′, D′, E′) and analysed directly in nucleoprotein gels. Less extensive centrifugation was necessary to catch the rapidly sedimenting complex from GH5/DNA (Figure 6B′), which turned out to be the complex of lower mobility in nucleoprotein gels in relatively pure form (Figure 6B, lane a). Separation of the more slowly sedimenting GH1 and GH5 complexes from free DNA (Figure 6D, E) required more extensive centrifugation (Figure 6D′, E′); after the shorter centrifugation (Figure 6B′ and

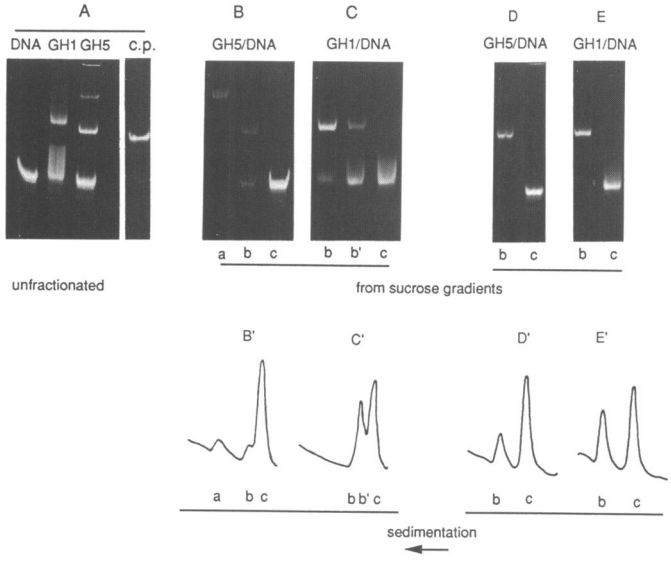

Figure 6. GH1 and GH5 bind cooperatively to ~146 bp DNA and form two distinct classes of complexes as judged by nucleoprotein gel electrophoresis. **A.** Comparison of complexes formed by GH1 and GH5 with ~146 bp DNA (input ratio 25%, w/w); unfractionated mixtures were analysed directly (alongside core particles, c.p.) in a 5% polyacrylamide gel which was stained with ethidium bromide. **B, C, D, E** (gels as in **A**) show the composition of the peaks from the corresponding 5–30% (w/w) sucrose gradients (**B', C', D', E'**; A_{280} profiles shown) when GH5/DNA or GH1/DNA mixtures (input ratio 25%, w/w) were fractionated; gradients were centrifuged in a Beckman SW 40 rotor at 29 000 rev./min for 16 h (B', C'), or at 40 000 rev./min for 18 h (D', E'), for optimal resolution of the fast-sedimenting (peak a) and slow-sedimenting (peak b) complexes, respectively.

C') these were ill-resolved from free DNA (see Figure 6B, lane b; and Figure 6C, lanes b and b').

The band migrating close to the position of free DNA in Figure 6A is noticeably smeared at the trailing edge in the case of the GH1/DNA mixture but sharp for GH5. This is also evident in Figure 6C, lane c (contrast B, lane c) and Figure 6E, lane c (contrast D, lane c). This, together with the finding by SDS-gel electrophoresis (not shown) of a small proportion of GH1 in the 'free DNA' peak in the sucrose gradients (Figure 6C', peak c; and Figure 6E', peak c), but not of GH5 in the corresponding peaks in Figure 6B' and D', suggests that GH1 binding to relatively short (~146 bp) DNA is less cooperative than GH5 binding (as well as less cooperative than GH1 binding to longer DNA, e.g. ~600bp, ~800 bp) for which the slower-moving peak in the gradient was indeed protein-free DNA.

The complexes containing GH1 or GH5 and ~200 bp DNA [40% (w/w) input ratio; ~6–7 mol G/mol DNA] taken from the sucrose gradients were also examined by electron microscopy. The single GH1/DNA peak contained mainly complexes of uniform thickness (~70 A; Figure 7A) together with a small proportion of free DNA (because the centrifugation conditions in this experiment had been chosen to optimize the separation of the rapidly-sedimenting GH5 complexes). Many of the complexes were about the length of free DNA and the DNA appeared to be uniformly coated with protein. Some complexes had short coated regions from which two DNA 'tails' emerged, as for the complexes with longer DNA (e.g. Figure 3C). The more slowly sedimenting GH5 complexes were generally similar in appearance (Figure 7B), although they sometimes appeared to be rather heterogeneous. The rapidly sedimenting GH5/DNA

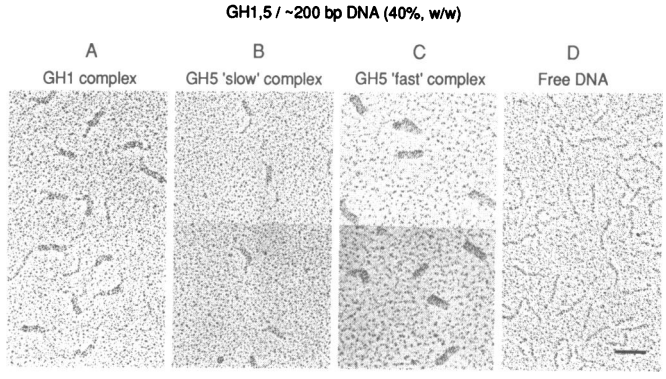

Figure 7. Electron microscopy of fractionated complexes of GH1 and GH5 with ~200 bp DNA. Complexes [formed at 40% (w/w) input] were single fractions taken from peaks in 5–30% sucrose gradients (c.f. Figure 6) centrifuged at 32 000 rev./min for 15 h, fixed with 0.1% glutaraldehyde, and dialysed to remove sucrose before electron microscopy . **A.** The GH1 complexes. **B.** The more slowly sedimenting GH5/DNA complex. **C.** The more rapidly sedimenting GH5 complex. **D.** Free DNA peak taken from the same gradient as in C. Scale bar represents 50 nm.

complexes (Figure 7C) were of strikingly uniform thickness (~150 Å) and of relatively uniform length which appeared to be about 85% that of free DNA (Figure 7D) taken from the same sucrose gradient.

Further characterization of the complexes

Chemical cross-linking indicates closely juxtaposed molecules of GH1 or GH5 in the cooperatively formed complexes. Material in the peaks from sucrose gradients containing GH1 or GH5/DNA complexes, together with unfractionated mixtures, were cross-linked (20) by addition of dithiobis(succinimidyl propionate) (DSP) and analysed by SDS/polyacrylamide gel electrophoresis (Figure 8). GH5 or GH1 free in solution gave no (in the case of GH1), or virtually no (GH5), intramolecular cross-linking under these conditions (protein at 12.5 mg/ml), as detected by Coomassie Blue staining (Figure 8A, lanes 2 and 5). Subsequent extensive silver staining of the gel confirmed that there was essentially no cross-linking of GH1(Figure 8A, lane 7) but revealed that a small proportion (~5%) of the GH5 was cross-linked into multimers (lane 9) [compared with about 50–60% under the same conditions in the presence of DNA (lane 10)]. GH5 does therefore appear to have a slight tendency to self-associate in solution, and this might be relevant to its behaviour bound to DNA and in chromatin. The increased electrophoretic mobility of both GH1 and GH5 on treatment with DSP in the absence of DNA (lanes 2 and 5; c.f. lanes 1 and 4) but not in its presence (lanes 3 and 6) is presumably due to reduction of the intrinsic positive charge on GH1 and GH5 due to acylation of lysine residues (some of which are protected in the presence of DNA), and possibly also to intramolecular cross-linking which might prevent complete unfolding of the proteins in SDS.

In striking contrast, GH1/DNA and GH5/DNA mixtures set up as described above gave a series of cross-linked bands $(G)_n$, (Figure 8A, lanes 3 and 6) indicating closely juxtaposed globular domains on the DNA in the cooperatively formed complexes in each case. A relatively low degree of cross-linking might be expected since a substantial proportion of the lysine residues are engaged in interaction with DNA [in GH5, ~5 of the 10 lysines,

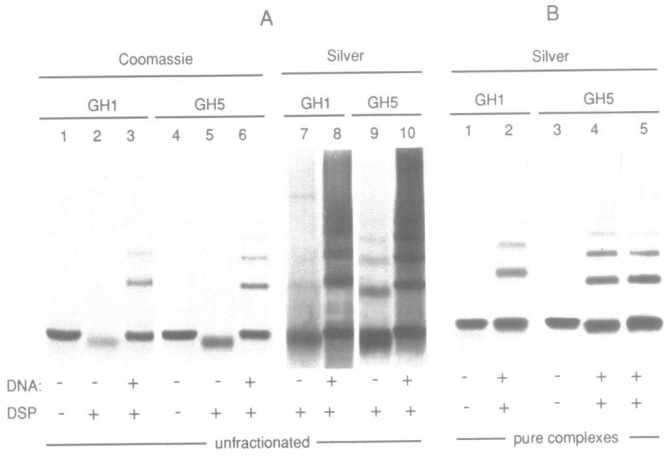

Figure 8. Cross-linking of unfractionated (**A**) and fractionated (**B**) GH1/DNA and GH5/DNA complexes with dithiobis(succinimidyl propionate) (DSP). **A.** Extensive cross-linking of GH1 and GH5 in the presence of DNA, but none in its absence in the case of GH1 and very little (not detectable by Coomassie staining) in the case of GH5. [Neither raising the pH to 8, nor a second addition of reagent, or a longer reaction time gave increased cross-linking (not shown).] Lanes 1 and 4, GH5 and GH1 alone; lanes 2 and 5, GH5 and GH1(at 12.5 μg/ml) treated with DSP; lanes 3 and 6, unfractionated GH1/~146 bp and GH5/~146 bp DNA mixtures [input ratio 25% (w/w)] treated with DSP (G, 12.5 μg/ml; DNA, 50 μg/ml). Cross-linking was carried out with 0.2 mg/ml DSP for 15 min and the products were analysed in an SDS/18%-polyacrylamide gel, which was stained with Coomassie Blue. The gel was then silver-stained heavily; lanes 7,8 and 9,10 are lanes 2,3 and 5,6 of the Coomassie-stained gel. **B.** GH1 and GH5 complexes fractionated in sucrose gradients show very similar cross-linking patterns. Complexes taken directly from sucrose gradients were cross-linked for 20 min with 0.3 mg/ml DSP and then analysed in an SDS/18%-polycrylamide gel, which was silver-stained directly. Lanes 1 and 3 contain GH1 or GH5 alone; lane 2 contains the cross-linked GH1 complex with ~200 bp DNA, and lanes 4 and 5 the cross-linked slower-sedimenting GH5 complex with ~200 bp and ~800 bp DNA respectively (the faster-sedimenting complex with 200 bp DNA gave an identical cross-linking pattern; not shown).

including the lysine most strongly protected from reductive methylation in H5 at its nucleosomal binding site (27), are (partially) protected in the presence of DNA (C.M. Wilson, R. Knopoff and J.O.T., unpublished)], but even so the bands extend up to n ~4.

Fractionated GH1/DNA complexes and the corresponding GH5/DNA complexes formed with ~200 bp DNA, when recovered from sucrose gradients and treated with DSP, also gave a series of cross-linked bands $(G)_n$ up to about $n = 4, 5$ in a silver-stained gel (Figure 8B, lanes 2 and 4), confirming the close juxtaposition of globular domains in the pure complexes. Identical results were obtained for complexes formed with ~800 bp DNA and cross-linked in parallel (lane 5) and for the GH5 fast-sedimenting complex (not shown).

The protein:DNA composition of complexes taken from sucrose gradients. If the DNA in the complexes were completely coated with protein it would be possible, from the protein:DNA ratio, to determine the site size for the globular domain, i.e. the number of base pairs 'occupied'. Attempts to determine the composition of the complexes will be confused (resulting in over-estimation of the number of base pairs occupied) both by the presence of free DNA co-existing with the complexes if the sedimentation condition are less than optimal for their resolution, and by regions of uncoated DNA within the complexes. As judged by electron microscopy the best sample for analysis seemed likely to be a GH1 complex formed at a relatively high input ratio and purified in a sucrose gradient. Complexes formed at an input ratio of 40% (w/w) GH1:DNA were therefore analysed.

The amount of protein associated with a known amount of DNA (estimated from the A_{260}) was determined by amino acid analysis after acid hydrolysis.This showed, firstly, that the weight ratio of GH1:DNA in the complexes is ~65%; this is roughly the input ratio at which loss of material occurs from the sucrose gradients by aggregation (e.g. Figure 2). Secondly, the values calculated for the number of base pairs per GH1 molecule in the complex were: 20.4 (for ~200 bp DNA), 21 (~400 bp DNA), 14.5–18.6 (~600 bp DNA), 21.9 (~800 bp DNA). If each complex contains two DNA duplexes, this would imply that two DNA segments of ~7–10 bp (~23.8 Å–34 Å) are 'occupied' by GH1, and would be entirely consistent with the diameter of ~29 Å reported for the globular domain (10, 11).

Analysis of the (sometimes) more heterogeneous slowly-sedimenting GH5 complex containing ~200 bp DNA , at an input ratio of 40% (w/w), gave a value (~21.8 bp/GH5 molecule) that was, perhaps surprisingly, similar to that for the GH1 complex. The relatively well defined fast-sedimenting GH5/~200 bp DNA complex (c.f. Figure 6B, lane a) in a single estimation, because of the difficulties in obtaining sufficient material, had a protein content roughly 2-fold higher (although this value should be regarded as tentative).

DISCUSSION

GH1 and GH5 bind cooperatively to DNA

We have shown that the isolated globular domains of H1 and H5 (GH1 and GH5), like the parent histones, bind cooperatively to DNA under conditions in which they show very little (in the case of GH5) or no (GH1) tendency to self-associate free in solution in the absence of DNA. Cooperative binding is evident at the macroscopic level from sedimentation analysis, electron microscopy and nucleoprotein gel electrophoresis, which show extensively coated protein-DNA complexes coexisting with free DNA, and at the microscopic level from protein-protein cross-linking which indicates molecules closely apposed on the DNA. At the ionic strength at which the complexes are formed, the isolated globular domains [at least of GH1 (28)] are probably not fully folded, but on binding to DNA folding is stabilized (8) and protein-protein interactions evidently permitted. Some cross-linking between globular domains was detected for intact H1 molecules cross-linked at high protein concentration (4 mg/ml) in the absence of DNA, typically with 1% formaldehyde for 18 hours, but in view of these extreme conditions the significance of this is unclear (29).

What is the extent of cooperativity in binding of the globular domains to DNA? The electron micrographs of unfractionated mixtures, e.g. for GH1 and ~800 bp DNA (Figure 3) at a low protein:DNA input ratio of 10% (w/w) (i.e. 6–7 molecules GH1 per DNA molecule), show some DNA molecules to be extensively and apparently uniformly coated, whereas the vast majority have no visible protein. Many of the complexes are well formed and appear to be roughly the length of the input DNA (and presumably contain two DNA duplexes approximately in register); in these, assuming close juxtaposition of globular domains which are ~29 Å in diameter, of the order of 90 [i.e. (~800×3.4)/29] molecules could be accommodated. This would imply a high degree of cooperativity in binding to DNA. As

discussed earlier, the limited array of globular domains that can be cross-linked is probably largely due to the low availability of many of the lysine e-amino groups through interaction with DNA. [The more extensive cross-linking of H1 molecules in H1/DNA complexes (8, 9) and chromatin (4, 5) is mainly due to the flanking basic domains, which are removed during isolation of the globular domain.]

With GH1 all the input DNA remains soluble over a wide range of protein:DNA ratios and sediments in sucrose gradients either in the peak of free DNA (or largely free DNA, in the case of very short DNA) or in the GH1/DNA complex peak. In contrast, GH5 sequesters a small proportion of the DNA into a complex with a higher protein:DNA ratio, leaving much of the DNA free of protein and a relatively small proportion in complexes resembling those formed with GH1. Whether the former complexes [presumably the thick complexes visualized with the electron microscope; Figure 4B (b)] are 'doubled-up' versions of fully coated thin complexes to which additional GH5 has bound, or (less likely) thin complexes with additional GH5, they suggest further interactions (GH5-DNA and/or GH5-GH5) that are not seen with GH1. Whether this difference between GH1 and GH5 has any biological significance, perhaps related to the known increased stability of the higher-order structure of H5-containing than the H1-containing chromatin (12) and possibly also to the low but finite tendency of GH5 to self-associate in solution found here, is unclear. Other differences between GH1 and GH5 also exist: GH5 has a more stable folded structure than GH1 (28), and it also binds more tightly to H1-depleted chromatin (30), possibly due to its higher arginine content which would result in stronger interaction with DNA (31).

The nature of the complexes

Both GH1 and GH5 appear to recruit pairs of DNA duplexes into complexes and it seems likely that an initial ternary complex then provides the framework for further (facilitated) cooperative binding of GH1 and GH5, as suggested earlier for complexes formed with H1 or H5 (8). The ability to bind more than one DNA molecule is evident for H1 in the nucleosome. H1 is located on the dyad axis (11, 32, 33) where it seals the two turns of DNA around the octamer, apparently through its globular domain (11), and is closely juxtaposed with the central turn of DNA as well as with the entering and exiting duplexes. The globular domain itself seems very likely to bind both the entering and exiting duplexes [since trimming of 10 bp from each end of 166 bp H1-containing chromatosomes causes H1 loss (34, 35), and the pause in digestion of nucleosomes at ~166 bp requires only the globular domain (11)], as well as the central DNA segment of the nucleosome, at the dyad axis (36). Lysine 85 in the globular domain of H5 interacts strongly with DNA in chromatin, and is thereby afforded protection against chemical modification (27); it lies in a region that exists as an apparently unstructured loop in isolated GH5 as deduced by NMR spectroscopy (37). A neighbouring region of the polypeptide chain, which is apparently helical in isolated GH5, has also been suggested to be significant in interaction with DNA, specifically the central turn around the nucleosome, at the dyad (38). Analysis of crystals of (a slightly extended version of) GH5 (39) may clarify the situation.

The H1(H5)/DNA complexes and the GH1(GH5)/DNA complexes are superficially similar (at the electron microscope level), mainly because the dominant feature of each is a pair of DNA duplexes (the 'tramlines') held together (presumably, in the case of the former complexes) by globular domains. However, globular domain (G-G) interactions are unlikely to occur in the H1(H5)/DNA ('thin') complexes because the average spacing of the histones along the tramlines appears to be substantially greater than would be expected if globular domains of diameter ~29 Å were in contact (9). The cooperative binding in the two cases thus appears to be different in at least some respects. In both cases provision of two DNA molecules in close juxtaposition will probably favour binding of further protein molecules within the ternary complex rather than on free DNA. However, in the case of globular domains there is also the possibility of G-G interactions, so that when successive globular domains bind, they do so in juxtaposition, whereas in the histone complexes other effects presumably operate (e.g. interactions between the highly cationic tails and DNA may result in local structural changes which favour binding of an adjacent histone molecule). The H1(H5)/DNA complexes are therefore likely to reflect the arrangement of the histones along the open, 10 nm, nucleosome filament (H1 or H5 bound to DNA duplexes near the nucleosome dyad through their globular domains and spreading their tails along the linker DNA) and the GH1(GH5)/DNA complexes to reflect additional (G-G) interactions in the 30 nm higher-order structure.

The globular domains of H1 (and H5) in chromatin

The results reported here lend support to the idea that interactions between the globular domains themselves in chromatin provide the basis for salt-dependent chromatin folding *in vitro*, and stabilization of the 30 nm filament *in vivo*. The globular domain may well be inside the solenoid, where G-G interactions would be easily accomodated, since it is inaccessible to ferritin-conjugated antibody (41) [see also (40)], although not to free antibodies (42, 43), presumably because the structure is 'breathing' and not tightly folded.

Attempts to identify long arrays of closely apposed H1or H5 globular domains within chromatin, by chemical cross-linking followed by excision of the globular domains from the histones with trypsin, has resulted in only a limited range of cross-linked products, mainly dimer with some trimer [(44); A.C. Lennard and J.O.T., unpublished observations)], in contrast with the much larger polymers of intact H1 and H5 under similar conditions (4, 5). However, since the cross-linking reagents react with lysine ϵ-amino groups, it seems likely that this reflects the much lower lysine content of the globular domain than of the flanking N- and C-terminal tails, and the protection of at least some of the lysine amino groups in the globular domain due to interaction with DNA (27), as is evidently also the case here for G/DNA complexes (Figure 8; and see text).

(Putative) G-G contacts in the chromatin higher-order structure might well be sensitive to changes in the interaction of the globular domain with the nucleosome and *vice versa*. It may be significant in this context that GH1-DNA interactions are disrupted, by some means, on activation of the *Drosophila hsp70* heat shock gene [as indicated by loss of a histidine-DNA contact in protein-DNA cross-linking experiments (45)]; GH1-GH1 contacts might concomitantly (as a cause or consequence) also be disrupted and, because of cooperativity, perhaps lead to unfolding of entire chromatin domains and to the DNase 1 sensitivity characteristic of transcriptionally competent chromatin that occurs without substantial H1 loss (46, 47). The results described here would be entirely compatible with this.

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Note added in proof

J.Widom and coworkers (P.H.Draves, P.T.Lowary and J.Widom, *J. Mol. Biol.*, submitted) also find that GH5 binds cooperatively to DNA (GH1 was not studied) and that the GH5/DNA complex contains two (or more) DNA duplexes, although fractionation into two distinct populations of complexes and free DNA in sucrose gradients, as shown here, was not achieved. We thank Dr Jonathan Widom for sending us the manuscript prior to publication.

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