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Identification of two DNA-binding sites on the globular domain of histone H5

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The nature of the complexes of histones H1 and H5 and their globular domains (GH1 and GH5) with DNA suggested two DNA-binding sites which are likely to be the basis of the preference of H1 and H5 for the nucleosome, compared with free DNA. More recently the X-ray and NMR structures of GH5 and GH1, respectively, have identified two basic clusters on opposite sides of the domains as candidates for these sites. Removal of the positive charge at either location by mutagenesis impairs or abolishes the ability of GH5 to assemble cooperatively in 'tramline' complexes containing two DNA duplexes, suggesting impairment or loss of its ability to bind two DNA duplexes. The mutant forms of GH5 also fail to protect the additional 20 bp of nucleosomal DNA that are characteristically protected by H1, H5 and wild-type recombinant GH5. They still bind to H1/H5-depleted chromatin, but evidently inappropriately. These results confirm the existence of, and identify the major components of, two DNA-binding sites on the globular domain of histone H5, and they strongly suggest that both binding sites are required to position the globular domain correctly on the nucleosome.

Keywords: chromatin/DNA binding/globular domain/ histone H5/mutagenesis

Introduction

Stabilization of chromatin higher order structure is dependent on histone H1 or its variants, such as H5 in nucleated (transcriptionally repressed) erythrocytes. Limit digestion of the nucleosome with micrococcal nuclease gives a core particle containing 146 bp of DNA and no H1; a metastable product in this pathway is the chromatosome (166 bp). If 10 bp of DNA are protected by H1 binding at each end of the chromatosome (Noll and Kornberg, 1977; Simpson, 1978) the simplest interpretation is that H1 interacts with the entering and exiting DNA in the nucleosome. The H1 (one molecule per nucleosome) would be located over the dyad axis, possibly contacting it (Allan *et al.*, 1980), thus also explaining the protection of the DNA at the dyad from digestion by DNase I (Staynov and Crane-Robinson, 1988). However, in the case of a nucleosome reconstituted on to DNA containing the *Xenopus* somatic 5S rRNA gene, protection of the additional 20 bp by H1 and H5 (Hayes and Wolffe, 1993) or GH5 (Hayes *et al.*, 1994) is asymmetric, and cross-linking of GH5 to DNA in the reconstituted nucleosome (Hayes *et al.*, 1994) has led to the proposal of an alternative location for H1 in that nucleosome (see Discussion).

The isolated globular domains of H1 or H5 (GH1, GH5) are sufficient to confer protection of 166 bp of DNA from micrococcal nuclease digestion (Allan *et al.*, 1980). The existence of two DNA-binding sites on the globular domain was suggested by the nature of GH1(GH5)–DNA complexes (Draves *et al.*, 1992; Thomas *et al.*, 1992) which resembled in appearance the H1(H5)–DNA complexes studied earlier (Clark and Thomas, 1986, 1988). The globular domain–DNA complexes consisted of 'tramlines' of two DNA duplexes bridged by an array of protein molecules; they probably arise by cooperative binding of GH1 or GH5 molecules to an initial ternary complex of one globular domain and two duplexes bound at two DNA-binding sites (Thomas *et al.*, 1992).

The similarity of the X-ray crystal structure of GH5 (Ramakrishnan *et al.*, 1993), and subsequently of the NMR structure of GH1 (Cerf *et al.*, 1994), to that of the sequence-specific DNA-binding proteins CAP (Schultz *et al.*, 1991) and HNF-3γ (Clark *et al.*, 1993) strongly suggested a primary DNA-binding site on GH5 and GH1 that corresponded to the DNA-binding sites on CAP and HNF-3γ, whose structures had been determined complexed with DNA (Ramakrishnan, 1994). The binding site on GH5 was consistent with existing biochemical evidence and included, for example, Lys85, which is strongly protected by interaction of H5 with the nucleosome (Thomas and Wilson, 1986). Replacement of Lys85 with glutamine or glutamic acid abolished correct nucleosome binding as judged by loss of protection of an additional 20 bp of DNA in the nucleosome from micrococcal nuclease digestion (Buckle *et al.*, 1992). The binding site also included His25, which could be cross-linked to DNA in chromatin (Mirzabekov *et al.*, 1989), and His62 which was shown to be cross-linked to DNA both in chromatin (Mirzabekov *et al.*, 1989) and in GH5–DNA complexes (F.A.Goytisolo, L.C.Packman and J.O.Thomas, unpublished). The other basic residues implicated in the primary DNA binding site were Lys69 and Arg73. The structure of GH5 revealed a second cluster of four positively charged residues (Lys40, Arg42, Lys52 and Arg94) on the opposite side of the GH5 molecule (Ramakrishnan *et al.*, 1993), some or all of which would be obvious candidates for components of a second DNA-binding site. Lys52 in this putative site and Lys69 in the proposed primary binding site were indeed protected from chemical modi-

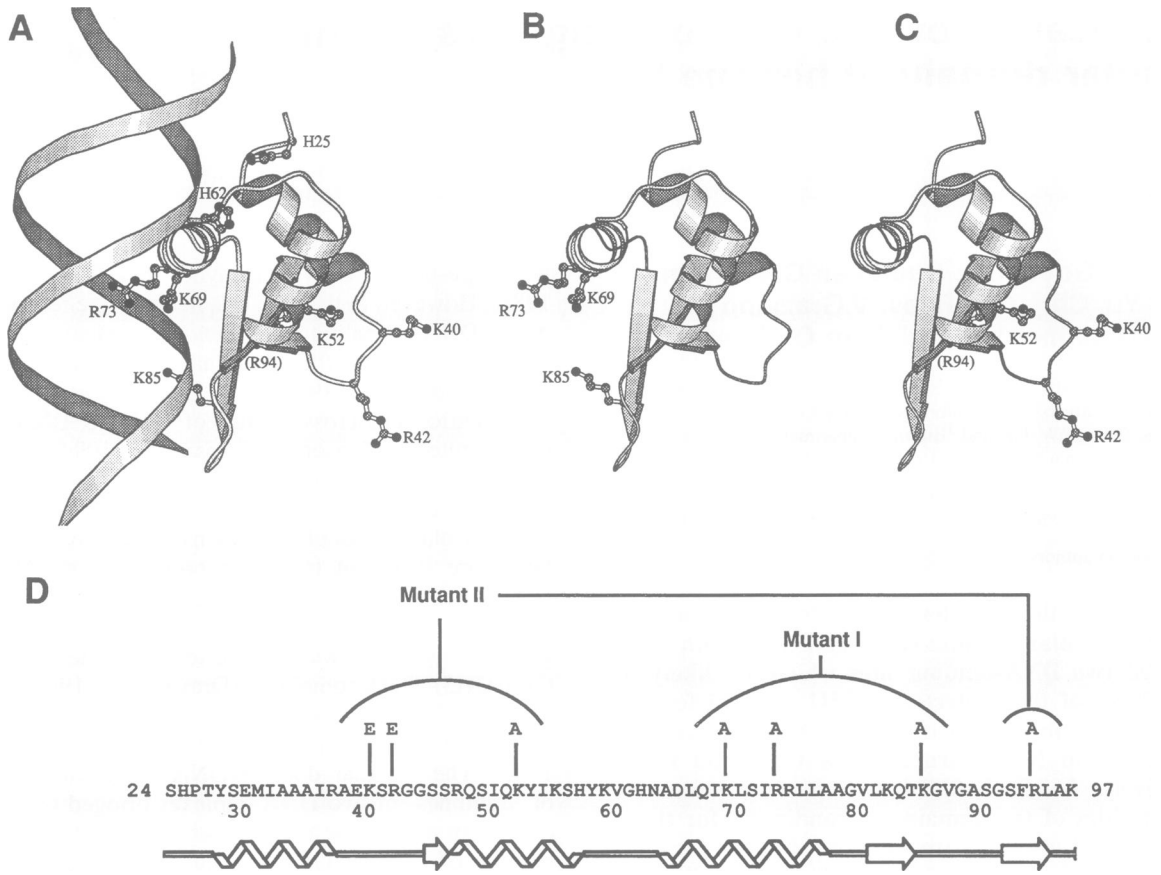

Fig. 1. The two clusters of putative DNA-binding residues in GH5. (**A**) Structure of GH5 showing the positions of the basic residues at both sites. (**B** and **C**) Basic residues at the proposed primary and secondary DNA binding sites, respectively. [Arg94, which is one of the residues changed to alanine in mutant II and mutant (I+II), is not visible in the view in A, B and C—and is therefore labelled (R94)—as it lies close to the C-terminus of GH5 which lies behind the molecule as shown.] (**D**) Amino acid sequence of recombinant GH5 showing the nature of the mutations (to alanine or glutamic acid) of the basic residues at the two sites.

fication by the association of H5 with chromatin, although to a lesser extent than Lys85 (Thomas and Wilson, 1986). Basic residues at the seven positions occupied by arginine or lysine in the primary and second DNA-binding sites are highly conserved in the H1 family (Crane-Robinson and Ptitsyn, 1989; Wells and McBride, 1989; Wells and Brown, 1991).

To determine whether the two putative DNA-binding sites were indeed functional, we removed the positive charges at one or both of the sites in GH5 by mutagenesis. Abolition of either positive cluster impaired or abolished the cooperative GH5–DNA complexes and, importantly, abolished chromatosome protection in the nucleosome. Residual cooperative GH5–DNA complex formation when only the primary cluster was mutated suggested that side chains of residues other than lysine and arginine were involved in DNA binding, His25 and His62 (see above) being likely candidates.

Results

Design, generation and characterization of mutants

The sequence corresponding to residues 24–97 of histone H5 (Briand *et al.*, 1980) in the T7-based expression vectors pET-3a (Studier *et al.*, 1990) or pET-13a (Gerchman *et al.*, 1994) was expressed in *Escherichia coli*. The recombinant globular domain (GH5) was purified, and its identity checked by N-terminal sequence analysis and electrospray ionization mass spectrometry.

Mutations resulting in the replacement of lysine/arginine by alanine or glutamic acid as indicated were introduced, using the polymerase chain reaction, either at the first putative DNA-binding site (site I) to give mutant I (K69A, R73A, K85A), or at the second such site (site II) giving mutant II (K40E, R42E, K52A, R94A), or at both sites [mutant (I+II)]. The substitutions to alanine were either in helices, which tolerate alanine well, or at positions which are occupied by alanine in the structure of HNF-3γ which is very similar to that of GH5 (<1.3 Å C-α root mean square difference between the corresponding parts of the two structures). The mutations to glutamic acid (E) were at residues that occurred in a solvent-exposed disordered loop in the GH5 structure, where alanine would have been a less obvious choice (although of course the loop might become structured on binding to DNA). The positions of the mutations in the GH5 tertiary structure and sequence are shown in Figure 1. The net effect of the mutations is to reduce the positive charge at site I from +3 to 0 and at site II from +4 to –2. The GH5 mutants were expressed, purified and characterized as for the wild-type recombinant GH5.

The mutants were checked for their structural integrity as judged by measurement of circular dichroism and for

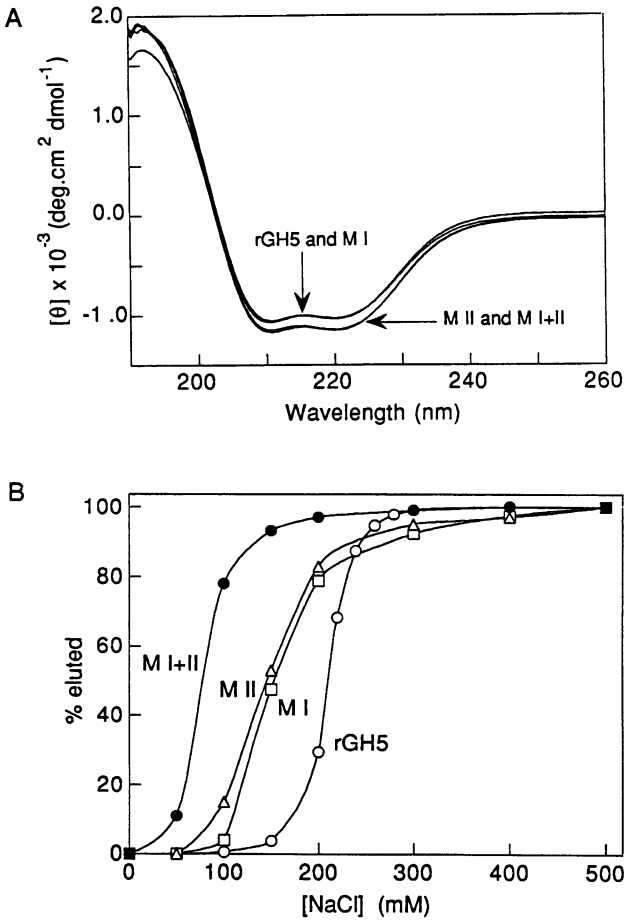

Fig. 2. Characterization of the folding and DNA-binding of wild-type and mutant recombinant GH5. (**A**) CD spectra of wild-type and mutant proteins in 10 mM Na phosphate pH 7.5. (**B**) Affinity for DNA–cellulose. The proteins were bound to DNA–cellulose at low ionic strength and eluted with increasing concentrations of NaCl (see Materials and methods). ○, wild-type recombinant GH5; □, mutant I; △, mutant II; ●, mutant (I+II).

their general ability to bind to bulk double-stranded DNA. Circular dichroism measurements in 10 mM Na phosphate pH 7.5, which is known to stabilize GH5 (de Petrocellis *et al.*, 1986), showed no loss of secondary structure relative to the wild-type GH5 (Figure 2A); the α-helix content, indicated by the negative ellipticity at 222 nm, was identical for wild-type GH5 and mutant I. The spectrum of mutant II was identical to that of mutant (I+II) but these mutants reproducibly showed slightly increased negative ellipticity compared with the wild-type protein and mutant I, indicating a slightly higher α-helix content, probably due to replacement of a basic residue (K52) in helix II with alanine, which is known to be a helix stabilizer (Horovitz *et al.*, 1992; Chakrabartty *et al.*, 1994 and references therein).

Salt-elution from double-stranded DNA–cellulose columns (Figure 2B) showed, not surprisingly, that the mutations at sites I and II both reduced the affinity for DNA (the mid-point of the elution curve occurred at ~200 mM NaCl for the wild-type protein and at ~150 mM for the mutants). The 'double mutant' [mutant (I+II)] bound more weakly still (elution mid-point at ~50 mM NaCl).

Cooperative binding to linear double-stranded DNA is impaired in the GH5 mutants as judged by sedimentation analysis and electron microscopy

Protein–DNA complexes were formed with wild-type or mutant recombinant GH5 at different histone:DNA input ratios and analysed by sedimentation through sucrose gradients, exactly as described in detail previously for GH5 produced by tryptic digestion of H5 ('tryptic GH5'; Thomas *et al.*, 1992). Complexes prepared with tryptic GH5 were prepared and analysed in parallel. The DNA was ~200 bp long, extracted from bulk chicken erythrocyte mononucleosomes. Figure 3B shows that the behaviour of the recombinant wild-type product is qualitatively very similar to that of the tryptic product (Figure 3A). In both cases cooperative binding of GH5 to DNA (Thomas *et al.*, 1992) was indicated by the coexistence of a rapidly sedimenting species (indicated with an asterisk), which contained DNA and GH5 and sedimented identically with the tramline ('thin') complexes reported previously (Thomas *et al.*, 1992), and free DNA which sedimented more slowly; there was also some pelleted material containing a relatively small proportion of the DNA but rich in GH5 which increased in amount with increasing protein:DNA input ratio, and is reflected in the decrease in the total amount of DNA sedimenting within the gradient. All of the input GH5 was bound to DNA; no free GH5 was left at the top of the gradient. The proportion of DNA sedimenting as a GH5–DNA complex at a particular protein:DNA input ratio (e.g. 40% w/w) was slightly less for the recombinant GH5 (residues 24–97) than for the tryptic GH5 (residues ~22–102; Thomas *et al.*, 1992) and we attribute this to additional basic residues in the latter which stabilize complex formation. Under these centrifugation conditions (40 000 r.p.m. for 18 h in a Beckman SW40 rotor) the tramline complexes (thin filaments) formed with GH5 and 200 bp DNA sediment within the gradient and the thick filaments formed by side-by-side association of the thin filaments travel to the bottom of the gradient (Thomas *et al.*, 1992). The complex peak observed here, which sediments more rapidly than free DNA, is identical in behaviour to the thin filaments.

Mutant I (Figure 3C) gave only a very small proportion of the rapidly sedimenting complex compared with the wild-type protein (Figure 3B), even at high protein:DNA ratios, suggesting that the ability to form stable cooperative tramline complexes had been severely impaired by removing the cluster of positive charges at site I. There was however, some protein–DNA aggregation, evident both as a broad, very rapidly sedimenting species near the bottom of the tube and as a pellet (see also below). When fixation of the protein–DNA mixture was carried out before centrifugation, the amount of complex increased slightly (data not shown), although it was still far less than that observed with wild-type recombinant GH5, suggesting that site I still retained some DNA-binding affinity despite loss of the positive charges, but that the complexes formed were unstable and partly dissociated during centrifugation.

The properties of mutants II and (I+II) were quite different. There was no trace of cooperatively formed GH5–DNA complexes (no fast-sedimenting peak in the gradient), even at high protein:DNA ratios (Figure 3D and E) and none was generated by fixation. All the GH5 was bound to DNA in the complex peak (data not shown)

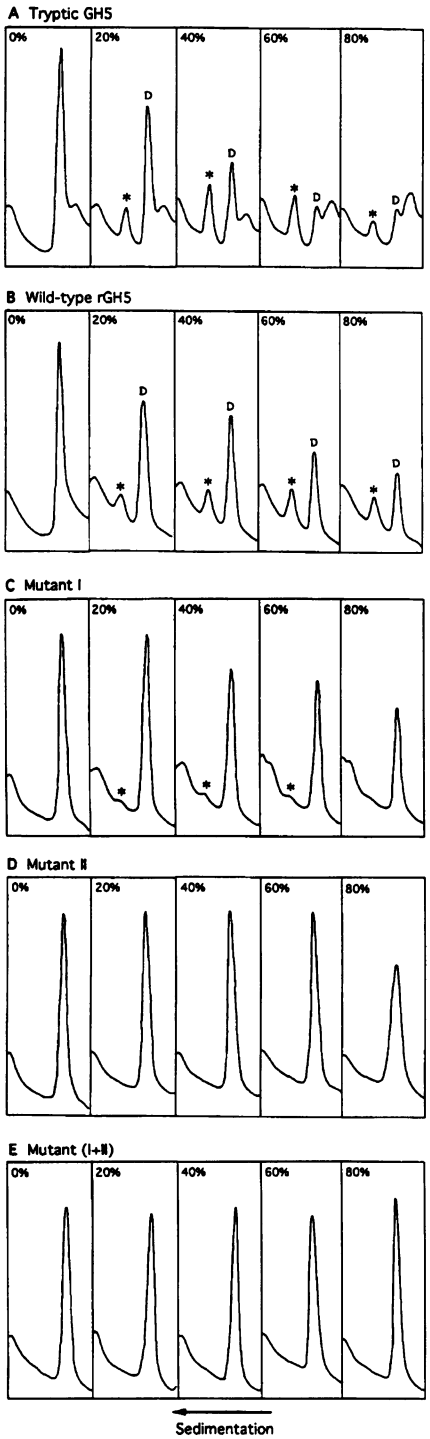

Fig. 3. Sedimentation analysis as a test of cooperative binding of the GH5 mutants. Protein–DNA complexes were formed in 5 mM triethanolamine–HCl pH 7.5, 5 mM NaCl at protein:DNA ratios of 20, 40, 60 and 80% (w/w) and centrifuged, together with DNA alone ('0%'), in a Beckman SW40 rotor at 40 000 r.p.m. for 16 h. (**A**) Tryptic GH5 (residues ~22–102 of H5); (**B**) wild-type recombinant GH5 (residues 24–97); (**C**) mutant I; (**D**) mutant II; (**E**) mutant (I+II). The asterisks (*) indicate rapidly sedimenting, cooperatively formed, protein–DNA complexes.

and there was none at the top of the gradient; there was also no pellet. The complex sedimented only slightly faster than naked DNA, like the complexes with dispersively bound H1 studied previously (Clark and Thomas, 1986). Removal of the positive charges at site II has therefore abolished the formation of cooperative (tramline) complexes.

Electron microscopy (not shown) confirmed that the rapidly sedimenting peaks for tryptic GH5, wild-type rGH5 (and mutant I; small peak) were the thin filaments of diameter ~7 nm reported earlier (Thomas *et al.*, 1992) whereas the single sedimenting peak given by complexes containing mutants II and (I+II) appeared to comprise only irregularly coated DNA. Unfractionated mixtures, which had not been subjected to sucrose gradient centrifugation, revealed the presence of some 'thick filaments' (from side-by-side association of thin filaments) for tryptic GH5 and rGH5, as reported earlier for the former (Thomas *et al.*, 1992). The thick filaments, which would have sedimented to the bottom of the gradients in Figure 3, were absent for mutants II and (I+II) but were found on the grid for mutant I, although less frequently than for wild-type recombinant GH5. The thick complexes confirm the conclusions drawn from the gradients, namely that cooperative tramline formation (which precedes thick filament formation) is clear for the tryptic GH5 (Thomas *et al.*, 1992), slightly less for the shorter recombinant GH5, and substantially impaired, although not wholly absent, for mutant I; mutants II and (I+II) give rise to no such complexes as judged by the absence of any thick complexes in the unfractionated samples.

It is clear from the sedimentation results and electron microscopy that two DNA-binding sites are necessary for the cooperatively formed regular 'tramline' complexes of GH5 and DNA (Thomas *et al.*, 1992). Removal of the four positive charges at site II (and the introduction of two negative charges), leaving site I intact, abolishes the tramline complexes completely. If site II is intact, but three positive charges are removed at site I, complex formation is impaired but not completely abolished, suggesting that site I may contain other DNA-binding residues, for example His25 and His62 (see above).

Binding to synthetic four-way junctions is abolished in the GH5 mutants

H5 and its globular domain show preferential binding to four-way junction DNA relative to linear double-stranded DNA (Varga-Weisz *et al.*, 1993, 1994). This may be another manifestation of the more stable binding of GH5 to structures containing two duplexes, which can be accommodated in the two proposed DNA-binding sites on GH5; this, of course, also occurs in the binding of successive GH5 molecules to 'tramlines', but in this case the process has to be initiated by ternary complex formation (one GH5 and two DNA duplexes) which is probably rate-limiting.

A ^{32}P-labelled four-way DNA junction used earlier for studies of HMG boxes (Teo *et al.*, 1995) was titrated with increasing amounts of wild-type GH5 and the various GH5 mutants in the presence of unlabelled sheared salmon sperm DNA as competitor, exactly as described (Varga-Weisz *et al.*, 1994). The results were clear-cut (Figure 4). Wild-type GH5 (Figure 4A) formed a discrete retarded complex (50% shift at 0.1 μM GH5) into which all the junction was converted at 0.2 μM protein. Further GH5 molecules appeared to bind, presumably to the arms, at higher concentrations, causing aggregation and a shift into

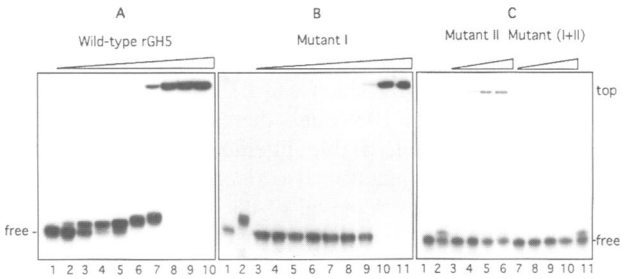

Fig. 4. Comparison of wild-type and mutant GH5 in structure-specific recognition of synthetic four-way junctions. ^{32}P-end labelled four-way junction was incubated at a final concentration of 0.27 nM with increasing amounts of wild-type or mutant GH5 and 50 μg/ml sonicated salmon sperm DNA as competitor. (**A**) Wild-type GH5 (lanes 2–10 contain 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 4.0, 8.0 and 16.0 μM protein); (**B**) mutant I [lanes 3–11 contain the same protein concentrations as in (A), lanes 2–10]; (**C**) mutant II (lanes 3–6 contain 2.0, 4.0, 8.0 and 13.0 μM protein) and mutant (I+II) (lanes 7–10 contain 2.0, 4.0, 8.5 and 17.0 μM protein). Samples were analysed in a 6% polyacrylamide Tris–glycine gel which was autoradiographed. Lane 1 in all cases contains DNA only. Lane 2 of (B), and lanes 2 and 11 of (C) contain wild-type GH5 at 0.2 μM and 0.1 μM respectively, as an 'internal control'.

the wells. In contrast, none of the three mutants [mutant I, II or (I+II)] gave any hint of a retarded complex (Figure 4B, lanes 3–11; Figure 4C, lanes 3–6 and 7–10). Mutant I gave some aggregate in the wells at high protein input, perhaps reflecting its residual weak ability to bind two duplexes since there was much less aggregate with mutant II and none with mutant (I+II).

It is clear that for structure-specific recognition of four-way junctions the two DNA-binding sites on GH5 must be present and intact. The structure-specific feature recognized in this case is probably the presence of two duplexes *per se*.

GH5 mutants bind to nucleosomes in chromatin but do not confer 166 bp 'chromatosome protection'

The pause in micrococcal nuclease digestion of nucleosomes at a DNA size of ~166 bp, corresponding to chromatosome protection (Noll and Kornberg, 1977; Simpson, 1978) can be restored to H1/H5-depleted chromatin by reconstitution with H1(H5) or tryptically generated globular domain alone (Allan *et al.*, 1980). Figure 5 (lanes 3 and 4) shows that the recombinant wild-type GH5 is also effective when reconstituted with H1/H5-depleted chromatin (tetranucleosomes). There was clear protection of ~166 bp DNA, in addition to the ~146 bp product resulting from further digestion of the chromatosome to the core particle. In contrast, none of the three mutants gave any 166 bp protection under these conditions, and digestion proceeded unimpeded to 146 bp (Figure 5, lanes 5 and 6, 7 and 8, and 9 and 10). There was likewise no protection of 166 bp by mutants I or II with three (rather than one) GH5 added per nucleosome (data not shown).

To determine whether the lack of protection was due to a failure of the mutant globular domains to bind to chromatin, samples were reconstituted with recombinant GH5 or the mutants, at 2 mol/nucleosome (to offset handling losses), and centrifuged through 5–30% sucrose gradients (Figure 6A). The peak fractions and the tops of the gradients (as well as the 'pellets') were analysed for

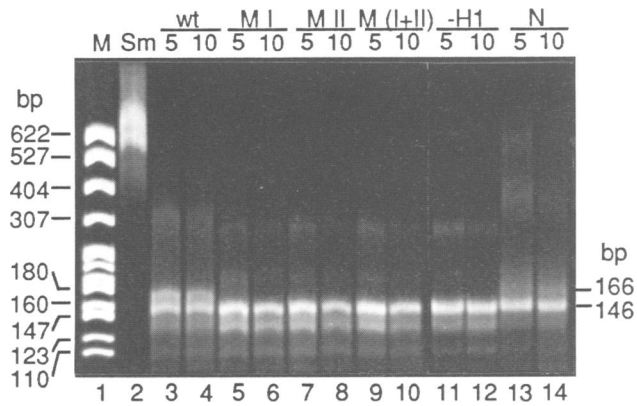

Fig. 5. Chromatosome (166 bp) protection is lost in the GH5 mutants. Tetranucleosomes depleted of H1 and H5 were reconstituted with wild-type or mutant GH5 (1 mol/nucleosome input) and digested with micrococcal nuclease for 5 min or 10 min. The DNA was extracted and analysed in a 6% polyacrylamide–TBE gel which was stained with ethidium bromide. Lanes 3 and 4, wild-type recombinant GH5; lanes 5 and 6, mutant I; lanes 7 and 8, mutant II; lanes 9 and 10, mutant (I+II). Lanes 11 and 12, and 13 and 14, contained respectively H1/H5-depleted tetranucleosomes and native tetranucleosomes, both digested with micrococcal nuclease as for the reconstitutes. Lane 1, pBR322/*Msp*I marker; lane 2, undigested H1/H5-depleted tetranucleosomes.

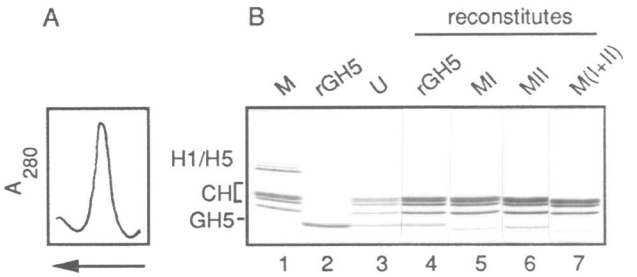

Fig. 6. GH5 mutants bind to H1, H5-depleted chromatin. Tetranucleosomes depleted of H1 and H5 were reconstituted with wild-type or mutant GH5 (2 mol/nucleosome input) in 10 mM TEA–HCl pH 7.5, 10 mM NaCl, 1 mM EDTA, 0.5 mM PMSF and centrifuged through 5–30% sucrose gradients containing the same buffer. (**A**) Gradient profile for wild-type GH5 reconstitute; all the other gradient profiles were identical. (**B**) The peak fractions analysed in an SDS/18% polyacrylamide gel which was stained with Coomassie blue; lanes 4, 5, 6 and 7 contain, respectively, the peak fractions from reconstitutes with wild-type recombinant GH5 (rGH5), mutant I (MI), mutant II (MII) and mutant (I+II) [M(I+II)]. Lane 3 contains an unfractionated reconstitute with rGH5 (2 mol/nucleosome) which was analysed directly in the gel and provides a standard for approximate estimation of the stoichiometry of GH5 binding. Lanes 1 and 2 contain respectively chicken erythrocyte total histones and rGH5, as markers.

protein content in SDS gels (to test for bound and unbound GH5). For the wild-type recombinant protein and mutants I and II (Figure 6B, lanes 4, 5 and 6, respectively) the single chromatin peak contained GH5 (recombinant wild-type or mutant) in similar amount, showing that the lack of chromatosome protection with the GH5 mutants was not due to lack of binding but, rather, failure to bind correctly. By comparison with lane 3, which contained a reconstitute of 2 mol GH5/nucleosome analysed directly in the gel without centrifugation, GH5 (wild-type and mutants I and II) was recovered at somewhat less than 1 mol/nucleosome in the peak fractions (lanes 4, 5 and 6); however, a lower than stoichiometric linker histone content of short oligonucleosomes is not unusual (e.g.

Caron and Thomas, 1981) and is largely due to end effects resulting from micrococcal nuclease invasion of the two terminal nucleosomes. In the case of mutant (I+II) (Figure 6B, lane 7), rather less (~50%) protein was bound to the chromatin peak than for rGH5 and mutants I and II, and this, together with the fact that no unbound protein was detected at the top of the gradient (or in the pellet), indicated that the binding was weaker than for mutants I and II (as it is also to DNA; see Figure 2B), and that mutant (I+II) was dissociating from the chromatin during sedimentation. There was some precipitation with the wild-type protein as expected (data not shown) since two GH5 molecules per nucleosome, rather than one, had been added, but there was no precipitation with any of the mutants.

Discussion

Identification of two DNA-binding sites on the globular domain

The starting point for the investigations described here was the circumstantial evidence that the globular domain of H1(H5) might make at least two physical contacts with nucleosomal DNA, at the exit and entry points, and the suggestion that the two DNA-binding sites strongly implicated in GH5 from previous biochemical evidence (Draves *et al.*, 1992; Thomas *et al.*, 1992) included two clusters of basic residues on opposite sides of the molecule. These comprised Lys69, Arg73 and Lys85 at the exposed 'primary' binding site (site I) on GH5, analogous to the single DNA-binding site on CAP and HNF-3γ (Ramakrishnan *et al.*, 1993), and Lys40, Arg42, Lys52 and Arg94 at the putative 'secondary' binding site (site II). We mutated the residues at the first site only (mutant I), or the second site only (mutant II), or both sites together [mutant (I+II)], either to alanine, or (in the case of two residues in site II which were in a disordered exposed loop in the GH5 structure) to glutamic acid. The mutations were largely without effect on the structure as judged from the CD spectra but (not surprisingly) lowered the affinity of the mutants for double-stranded DNA–cellulose. The choice of glutamic acid rather than alanine for two of the residues at site II was in order to preserve the polar character of the loop. The consequences will depend on the roles of the basic residues that have been thus substituted. If they did not interact with DNA they (and the glutamic acid residues that replaced them) would be solvated, and behave essentially like neutral residues; if they interacted electrostatically with DNA phosphates, the net charge change of –2 (+1 to –1) would lead to repulsion, and could lead to the complete loss of function at site II observed here when the four basic residues are mutated. It is, of course, possible that other residues at site II might also be involved in weak interactions with DNA (cf. His25 and His62 at site I), and that these residual interactions might be abolished in the site II mutant due to a strong repulsive interaction from substitution of a basic DNA-binding residue with glutamic acid.

Sedimentation experiments strongly indicated that the two positive clusters were necessary to form the cooperative tramline complexes formed when GH5 binds to DNA (Draves *et al.*, 1992; Thomas *et al.*, 1992). Mutants II and (I+II) formed no such complexes. Their formation was also impaired for mutant I, although not abolished, possibly due to stabilization of the complexes by His25 and His62 [which can be cross-linked to DNA (Mirzabekov *et al.*, 1989) and therefore must effectively contact it] at site I. Stable interaction of GH5 with a synthetic four-way junction also clearly required both sites I and II to be intact; removal of the charges at either site abolished binding. These studies together therefore provide clear evidence for the presence of at least two functional DNA-binding sites on the globular domain of H5, corresponding to the two clusters of positive charges identified earlier (Ramakrishnan *et al.*, 1993). The two sites are distinct and well separated, in that they are physically on different faces of GH5. Site I is clearly analogous to the DNA-binding site of HNF-3γ and other sequence-specific DNA-binding proteins, whereas the precise nature of site II and its mode of interaction with DNA remain to be determined.

The most likely explanation for the preference of the globular domains of H1 and H5 for binding two duplexes, or to four-way junctions, is that this mimics their natural binding site on the nucleosome, where two (or three) duplexes are in close proximity. That the two DNA-binding sites on GH5 identified here are indeed involved in correct binding of the globular domain to the nucleosome is shown by the chromatosome protection assays. Although all the mutants were able to bind to H1/H5-depleted oligonucleosomes, as shown by co-sedimentation in sucrose gradients [mutant (I+II) binding least strongly], unlike the wild-type protein they offered no stabilization of two turns of DNA around the histone octamer, which would be reflected in the protection of ~166 bp of DNA. This shows clearly that, in the native chromatin context, the globular domain of H5 interacts with the nucleosome through at least two distinct DNA binding sites, and that these sites are composed of, or at least include, the basic residues that have been mutated. It is likely that similar DNA-binding sites occur also in H1, in view of the structural similarity between GH5 (Ramakrishnan *et al.*, 1993) and GH1 (Cerf *et al.*, 1994), and also in H1 variants and subtypes generally, given the high degree of conservation of all the amino acids of H5 mutated in this study. They are all almost completely conserved in the H1 family as basic residues (Crane-Robinson and Ptitsyn, 1989; Wells and McBride, 1989; Wells and Brown, 1991). Figure 7 shows a representative set of globular domain sequences from a variety of species.

Relevance to the location of the globular domains of linker histones in the nucleosome

Binding of GH5 over the dyad axis of the nucleosome, possibly but not necessarily contacting it, such that it interacts with the entering and exiting DNA duplexes, is probably the simplest way of accounting for protection of 10 bp at each end of chromatosomal DNA (Simpson, 1978) and for the footprinting results for H1 in dinucleosomes (Staynov and Crane-Robinson, 1988). Based on the preferential occurrence of particular base steps (ApG and GpG) at one terminus of chromatosomal DNA (and their complements at the other), and the known cross-linking of H1 to DNA within this region (Belyavsky *et al.*, 1980), it was suggested (Muyldermans and Travers, 1994) that there might be some sequence specificity in H1 binding,

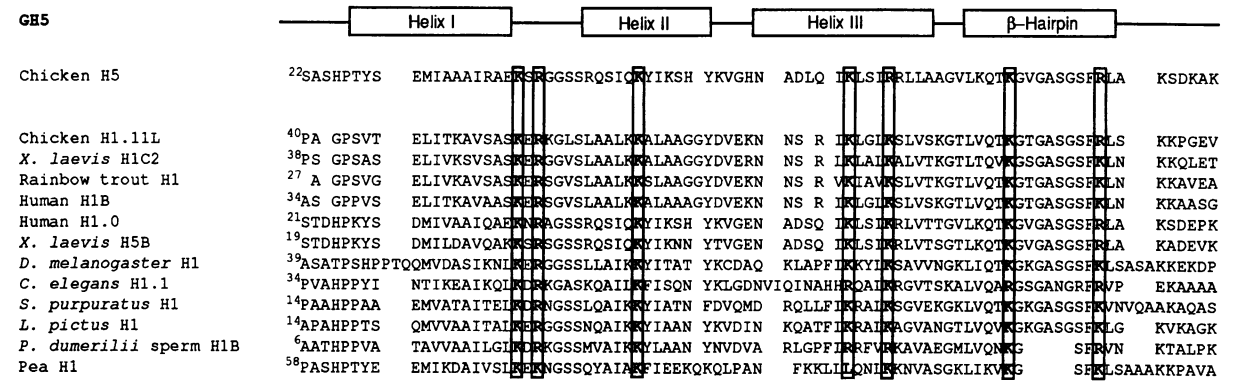

Fig. 7. Sequence alignment of the globular domains of linker histones from a representative range of species showing (boxed) conserved basic residues in the putative DNA binding sites I and II (see text). The secondary structure of GH5 (Ramakrishnan *et al.*, 1993) is shown schematically at the top. Numbers at the beginning of each line indicate amino acid positions within the parent proteins. The limits of the sequences shown in the alignment are those corresponding to the limits of GH5 produced by tryptic digestion (Thomas *et al.*, 1992). Gaps are introduced for optimal alignment. Protein sequences are as given by Wells and McBride (1989) and Wells and Brown (1991).

and in particular that the conserved sequences might be recognized in the major groove by the putative primary DNA-binding site (helix III) in GH5. The second DNA-binding site might (in some way) contact the other terminus (Belyavsky *et al.*, 1980) close to, or at a distance from, the dyad (Muyldermans and Travers, 1994). Neutron scattering results have shown that in the 30 nm chromatin filament the centre of mass of H1 is at approximately the same radial location as the inner face of the nucleosome (Graziano *et al.*, 1994). If the linker DNA is assumed to be on the inside of the 30 nm filament, the simplest model would be one in which GH5 indeed interacted with the entry and exit strands of DNA close to the dyad. However, since the flanking N- and C-terminal tails of H1 comprise over half the mass of the protein, models that place the globular domain further away from the dyad (see below) are not ruled out by these data.

In contrast to the suggestions of a pseudo-symmetrical location for the globular domain, protein–DNA cross-linking of a nucleosome reconstituted with GH5 on the *Xenopus laevis* 5S rRNA gene, which contains a strong octamer positioning signal, suggests that GH5 is not located over the dyad axis, but ~65 bp away, either on the outside of the DNA superhelix at 3.5 nm from the dyad, or on the inside, in a 'hole' in the octamer, which would place GH5 closer to the dyad axis (Hayes *et al.*, 1994; Pruss *et al.*, 1995). However, there is no *a priori* requirement in such models for interaction of GH5 with two duplexes, or therefore for the two binding sites on the globular domain which our results strongly indicate, although in the external location GH5 might perhaps be able to interact also with the adjacent DNA gyre on the body of the nucleosome core. It is proposed (Hayes *et al.*, 1994; Pruss *et al.*, 1995) that an off-dyad location for the globular domain, and hence for the intact linker histone (if generally true and not unique to the 5S reconstituted nucleosome) could still account for protection by linker histones of the DNA at the ends of the chromatosome, whether symmetrical (Noll and Kornberg, 1977; Simpson, 1978; Pehrson, 1989; Muyldermans and Travers, 1994) or non-symmetrical (Hayes and Wolffe, 1993), by transmitted conformational changes; the protection would arise from contact of the DNA with core histones, and indeed such contacts have been reported for specialized histone variants (Hill and Thomas, 1990; Lindsey *et al.*, 1991). However, it remains to be seen if an off-dyad location, with no *a priori* requirement for two DNA-binding sites on GH1, holds generally for bulk DNA sequences, or may rather be a feature of (certain) reconstituted mononucleosomes. The only evidence for it so far is protein–DNA cross-linking of the 5S reconstituted nucleosome (Hayes *et al.*, 1994; Pruss *et al.*, 1995), together with lack of protection by H5 at the dyad as judged by hydroxyl radical and DNase I footprinting of the reconstituted nucleosome (Hayes *et al.*, 1994). The cross-linking result is clear but, inevitably with this method, not easy to quantitate, and the possibility that the reconstitute contains a mixture of major and minor species, only the latter of which has a purine suitably disposed to lead to cross-linking to a basic side-chain [of lysine or histidine (Nacheva *et al.*, 1989)] in GH5 cannot be excluded. Indeed, it has been shown that histone octamers can occupy multiple positions on multiple tandem copies of the sea urchin 5S sequence (Dong *et al.*, 1990; Pennings *et al.*, 1994) as well as on two tandem copies of the *Xenopus* 5S gene (Ura *et al.*, 1995). It is also possible that although the 5S sequence is a good octamer-positioning sequence it does not contain sequences optimal for H1-containing chromatosomes (the existence of such sequences may be inferred from the results of Muyldermans and Travers, 1994) so that although H1 binds to a reconstituted 5S nucleosome particle in preference to free DNA (Hayes and Wolffe, 1993) the binding may not be typical of H1 binding to bulk nucleosomes. In this connection we note the recent report of a high incidence of a 'chromatosome positioning sequence' in a bulk population of chromatosomes (Travers and Muyldermans, 1996) and that this sequence is not present in the 5S DNA in the relevant position in relation to the dyad.

In summary, the evidence presented here suggests that the two putative DNA-binding sites indicated by the GH5 crystal structure correspond functionally to the two DNA-binding sites on the globular domain that were strongly suggested by our earlier studies of cooperative GH5–DNA complexes. Each site includes a cluster of highly conserved basic residues, although we cannot rule out the possibility that other residues also contribute to these sites, and indeed there is evidence for this in the case of site I.

Whereas the nature of site I can be reliably inferred from the structural similarity between GH5 and other DNA-binding proteins, the nature and extent of site II, and indeed whether all four basic residues are involved in one functional site, can only be determined by more detailed mutagenesis and biochemical studies, which are in hand or, for example, by co-crystallization and X-ray structure determination. It is clear, however, that the two sites are functional in chromatin and *both* are required for chromatosome stabilization. There may conceivably be other, additional, DNA-binding sites but the requirement for at least two sites places constraints on proposals for the location of the globular domain of H1 or H5 in the nucleosome.

Materials and methods

Wild-type and mutant recombinant GH5

The sequence corresponding to residues 24–97 of histone H5 (Briand *et al.*, 1980) was subcloned from a plasmid containing a genomic clone of H5 (Krieg *et al.*, 1983) into the T7-based expression vectors pET-3a (Studier *et al.*, 1990) or pET-13a (Gerchman *et al.*, 1994) and expressed in *E.coli*. Mutations were introduced into the wild-type gene by using the 'megaprimer PCR' method (Landt *et al.*, 1990). DNA constructs were all checked by sequencing the double-stranded plasmids with Sequenase (US Biochemicals) using the protocols provided by the manufacturer.

Cells with the appropriate mutant gene were grown to an OD_{600} of 0.8, induced with 0.5 mM isopropylthiogalactoside, harvested and stored at –70°C. Protein was purified from the cells as follows, all at 0–4°C and using buffers containing 50 mM phenylmethylsulfonyl fluoride (PMSF) and 50 mM benzamidine. Cells were thawed and suspended in five volumes of lysis buffer (50 mM Tris–HCl pH 8.0, 1 mM EDTA) and treated with lysozyme at a final concentration of 1 mg/ml for 1 h on ice. Cells were lysed by the addition of sodium deoxycholate to 0.08% (w/v). The resulting viscosity was reduced by treatment with DNase I (2 μg/ml) for 15 min in the presence of 10 mM $MnCl_2$ and 10 mM $MgCl_2$. The extract was then centrifuged at 12 000 r.p.m. in a Sorvall SS34 rotor, and the supernatant was loaded on to a S-Sepharose Fast-Flow (Pharmacia) column equilibrated in lysis buffer. The column was eluted with a gradient of 0–1 M NaCl in lysis buffer and fractions containing GH5 were pooled, concentrated and applied to a Sephacryl S-100 HR (Pharmacia) gel filtration column equilibrated in lysis buffer containing 0.2 M NaCl. The fractions from this column containing GH5 were essentially pure as judged by Coomassie staining of 8–25% polyacrylamide–SDS gels.

Expressed proteins were analysed by N-terminal sequencing (five residues) using an Applied BioSystems 477A Pulsed Liquid Sequencer and by electrospray mass-spectrometry on a VG BioQ quadrupole instrument (Weir *et al.*, 1993). Proteins concentrations were determined by amino acid analysis, using the known amino acid sequence (Clark and Thomas, 1986).

Tryptic GH5

GH5 comprising residues ~22–102 of H5 was produced by tryptic digestion of H5, and characterized as described previously (Thomas *et al.*, 1992).

DNA–cellulose binding

The proteins (30 μg) were adsorbed on to DNA–cellulose (Sigma; 4 mg calf thymus double-stranded DNA/g cellulose) equilibrated with 5 mM triethanolamine (TEA)–HCl pH 7.5, 5 mM NaCl, and eluted with stepwise addition of buffer containing increasing concentrations of NaCl (50–500 mM) as described previously (Hill *et al.*, 1991). Eluted proteins were precipitated with trichloroacetic acid, analysed in SDS/18% polyacrylamide gels which were then stained with Coomassie brilliant blue R250 (Thomas and Kornberg, 1978), and quantitated by scanning densitometry using a Molecular Dynamics 300S laser scanning densitometer.

Circular dichroism

The proteins were diluted to a final concentration of 0.12 mg/ml in buffer containing 10 mM Na phosphate pH 7.5. All spectra were recorded at ~20°C over the range 190–260 nm (with measurements every 0.5 nm) on a Jobin-Yvon CD6 spectropolarimeter in cuvettes with a 1 mm path length; they represent the average of six scans which were subsequently processed for baseline subtraction and smoothing using the manufacturer's software. All spectra are presented as molar ellipticity, [θ], based on the appropriate mean residue weights [108.36 for wild-type GH5, 105.68 for mutant I, 106.09 for mutant II and 103.39 for mutant (I+II)].

GH5–DNA complexes

Complexes were formed in 5 mM TEA–HCl pH 7.5, 5 mM NaCl, 0.5 mM PMSF at various protein:DNA input ratios (20–80% w/w) using ~200 bp DNA [extracted from chicken erythrocyte mononucleosomes (Clark and Thomas, 1986)] at a final concentration of 25 μg/ml (Thomas *et al.*, 1992). They were analysed by centrifugation through 5–30% (w/v) sucrose gradients containing the same buffer, which were centrifuged at 40 000 r.p.m. for 16 h at 4°C in a Beckman SW40 rotor. The peak, top and bottom ('pellet') fractions were analysed for protein content in SDS/18% polyacrylamide gels (Thomas and Kornberg, 1978).

Binding to four-way junctions

The binding of wild-type and mutant GH5 to a ^{32}P-labelled synthetic four-way junction DNA (Teo *et al.*, 1995) in the presence of an excess of sonicated (~800–1200 bp) salmon sperm DNA (Sigma) was analysed essentially as described by Varga-Weisz *et al.* (1994), using 6.5% polyacrylamide gels containing Tris–glycine pH 8.9. Gels were run at 50 V for 6 h at 4°C, vacuum-dried and autoradiographed for ~15 h at –80°C with intensifying screens.

Reconstitution of GH5 and mutants on to H1/H5-depleted oligonucleosomes

Chromatosome protection. Tetranucleosomes were isolated from a micrococcal nuclease digest of chicken erythrocyte chromatin by size-fractionation in a 5–30% (w/v) sucrose gradient (Bates *et al.*, 1981), then dialysed, concentrated, and stripped of H1 and H5 by recentrifugation through a similar gradient containing 0.5 M NaCl (cf. Butler and Thomas, 1980). The stripped oligonucleosomes were dialysed into 5 mM TEA–HCl pH 7.5, 5 mM NaCl. Wild-type or mutant GH5 were added at 1 mol/nucleosome (based on the A_{260}) to 80 μl of oligonucleosomes at A_{260} = 2, followed by $CaCl_2$ to 1 mM and micrococcal nuclease (5 U). Digestion was allowed to proceed for 5 min or 10 min at 23°C and then stopped by the addition of 0.1 M EDTA pH 7 to 10 mM and chilling on ice. The DNA was extracted with chloroform/isoamyl alcohol, ethanol precipitated and analysed in a 6% polyacrylamide gel containing TBE (89 mM Tris, 89 mM boric acid and 2 mM EDTA) which was then stained with ethidium bromide and photographed under short-wavelength UV transillumination.

Sedimentation analysis. Wild-type or mutant GH5 was added at 2 mol/nucleosome to 0.4 ml samples of dialysed, H1/H5-depleted tetranucleosomes (see above) at A_{260} = 4.4. The samples were incubated for 30 min at 4°C and then analysed in 5–30% (w/v) sucrose gradients containing 10 mM TEA–HCl pH 7.5, 10 mM NaCl, 0.5 mM PMSF, centrifuged in a Beckman SW40 rotor at 4°C for 12 h at 30 000 r.p.m. The gradients were fractionated, and monitored at 280 nm; material from the peak fractions and from the top and bottom ('pellet') of the gradient was analysed in SDS–18% polyacrylamide gels (Thomas and Kornberg, 1978).

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