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Competition between HMG-I(Y), HMG-1 and histone H1 on four-way junction DNA

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ABSTRACT

High mobility group proteins HMG-I(Y) and HMG-1, as well as histone H1, all share the common property of binding to four-way junction DNA (4H), a synthetic substrate commonly used to study proteins involved in recognizing and resolving Holliday-type junctions formed during *in vivo* genetic recombination events. The structure of 4H has also been hypothesized to mimic the DNA crossovers occurring at, or near, the entrance and exit sites on the nucleosome. Furthermore, upon binding to either duplex DNA or chromatin, all three of these nuclear proteins share the ability to significantly alter the structure of bound substrates. In order to further elucidate their substrate binding abilities, electrophoretic mobility shift assays were employed to investigate the relative binding capabilities of HMG-I(Y), HMG-1 and H1 to 4H *in vitro*. Data indicate a definite hierarchy of binding preference by these proteins for 4H, with HMG-I(Y) having the highest affinity (K_d ~6.5 nM) when compared with either H1 (K_d ~16 nM) or HMG-1 (K_d ~80 nM). Competition/titration assays demonstrated that all three proteins bind most tightly to the same site on 4H. Hydroxyl radical footprinting identified the strongest site for binding of HMG-I(Y), and presumably for the other proteins as well, to be at the center of 4H. Together these *in vitro* results demonstrate that HMG-I(Y) and H1 are co-dominant over HMG-1 for binding to the central crossover region of 4H and suggest that *in vivo* both of these proteins may exert a dominant effect over HMG-1 in recognizing and binding to altered DNA structures, such as Holliday junctions, that have conformations similar to 4H.

INTRODUCTION

Recognition and alteration of DNA structure plays a significant biological role in regulating transcription, replication, genetic recombination and repair in both prokaryotes and eukaryotes. In eukaryotes the overall folded structure of DNA is determined primarily by its association with 'core' and H1-type linker histones to form nucleosomal chromatin (1,2), but with additional and often localized contributions being made by both transcription factors and ancillary chromatin proteins such as members of the high mobility group (HMG) proteins.

Histone H1 (M_r ~23 kDa) and its variants (e.g H5, Hl°, etc.) are commonly referred to as linker histones because of their ability to preferentially bind to the linker DNA of nucleosomes (1–4), although their exact placement with respect to the nucleosome core particle is still unknown (5–13). The crystallographic structure of the globular domain of histone H5 (6) indicates that its three α-helices assume a 'winged' configuration with two potential regions of contact with DNA, a prediction confirmed by recent mutagenesis studies (11). The linker histones play a pivotal role in chromatin compaction (recently reviewed in 4) and can exert either a positive or negative role on gene transcription both *in vitro* (14,15) and *in vivo* (16,17). H1 binds to DNA in a sequence-independent fashion yet has been shown to preferentially bind to supercoiled plasmids over either linear or relaxed DNAs (18–20). In addition, H1 is known to preferentially bind to certain altered DNA structures such as four-way junctions (4H) (21) [The nomenclature for four-way junction used here is that defined in Lilley *et al*. (22) and in Materials and Methods.] H1 not only recognizes certain altered forms of DNA but is able to induce changes in DNA structure, as demonstrated by its ability to both unwind naked DNA (23,24) and to compact chromatin (1–3).

HMG-1/-2 proteins (M_r ~25 kDa) are the largest and most abundant of the HMG proteins and have been implicated in both positive and negative regulation of gene transcriptional activity *in vitro* and *in vivo*, but their precise biological functions are still uncertain (reviewed in 25). They interact with DNA through their two DNA binding domains, known as HMG-1 boxes, a conserved set of amino acids folding into three α-helices forming an L-shaped structure (26,27). HMG-1/-2 proteins bind to the minor groove (28) of double-stranded DNA in a sequence-independent manner (25). HMG-1/-2 binding affects DNA structure by inducing bends in linear substrates (29–31) and by introducing supercoils into topologically constrained molecules (32–34). Recently HMG-1 has been shown to preferentially bind to altered DNA structures which contain sharp bends, such as 4H (35) and those found in cisplatin–DNA adducts (36).

HMG-I (M_r ~11.7 kDa) and HMG-Y (M_r ~10.5 kDa) are isoform proteins produced by alternative splicing of mRNA transcripts from a single gene (37,38). For convenience and to distinguish them from HMG-1/-2 proteins, members of this family will be referred to as HMG-I(Y). HMG-I(Y) proteins have

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been demonstrated to be involved in both positive and negative gene regulation, possibly by functioning as accessory 'architectural transcription factors' (reviewed in 25). HMG-I(Y) proteins preferentially bind to the minor groove of A·T-rich B-form DNA by recognition of substrate structure rather than nucleotide sequence (25,39–44). *In vivo* HMG-I(Y) recognizes the structural features of DNA through its DNA binding domains, known as A·T hooks, which also bind to altered DNA structures such as 4H (45), those found on the front face of native (random sequence) nucleosome core particles (46) and on the surface of nucleosomes reconstituted from defined sequence DNAs (40). In addition to recognizing structure, HMG-I(Y) has the ability to induce structural changes in DNA substrates. For example, HMG-I(Y) can unwind DNA, induce both positive and negative supercoils into topologically constrained plasmid DNAs (47) and introduce bends and/or other distortions into linear substrate molecules (48; G.Schroth and R.Reeves, unpublished data).

Even though the three-dimensional structures of the DNA binding domains of the H1/H5 linker histones (6), of the HMG-1/-2 proteins (26,27,31) and of HMG-I(Y) (49–51) are all quite different, they share many common DNA binding characteristics, including the ability to bind 4H substrates. The structure of 4H has been rigorously analyzed and many structural features elucidated and characterized (reviewed in 52).

Although synthetic 4H substrates are not associated with core histones, they do contain two converging DNA strands which have been proposed to imitate the structure of DNA found at, or near, the entrance and exit points of the nucleosome and have therefore been suggested as a simple model system for studying chromosomal proteins that bind to such regions (4,21,30,53). Perhaps more importantly, 4H simulates *in vivo* Holliday junctions and has therefore been used extensively as an intermediate substrate for genetic recombination events (52,54–58).

With these potential biological contexts in mind, we performed quantitative *in vitro* binding and competition experiments to investigate the relative strengths and specificities of the physical interactions of HMG-I(Y), HMG-1 and H1 proteins with 4H. Our results demonstrate that HMG-I(Y) binds most tightly to 4H with a K_d of ~6.5 nM, followed by H1 and HMG-1 with K_d values of ~16 and ~80 nM respectively. We also demonstrate that HMG-I(Y) is far more effective in competing with H1 than is HMG-1 for binding to 4H substrates. Hydroxyl radical footprinting of HMG-I(Y) on 4H indicates that the protein protects 4H at the crossover, thereby demonstrating for the first time that HMG-I(Y) preferentially binds to the altered structure at the center region of the 4H structure. Our findings suggest that HMG-I(Y) and H1 exert a dominant effect over HMG-1 in binding to altered DNA structures *in vivo* and therefore have important implications for their possible biological roles in regulation of chromatin structure and function, as well as for participation in recombination and integration events.

MATERIALS AND METHODS

Preparation of oligonucleotides

Oligonucleotides 1–4 previously described by Bianchi (35) were used to create 4H:
Leg 1, CCCTATACCCCTGCATTGAATTCCAGTCTGATAA;
Leg 2, GTAGTCGTGATAGGTGCAGGGGTTATAGG;
Leg 3, AACAGTAGCTCTTATTCGAGCTCGCGCCCTATCACGACTA;
Leg 4, TTTATCAGACTGGAATTCAAGCGCGAGCTCGAATAGAGCTACTGT.
The oligonucleotides were synthesized using an Applied Biosystems DNA synthesizer and subsequently purified by electrophoresis on a denaturing 1.5 mm thick, 1× TBE, 16% polyacrylamide (19:1 bisacrylamide) gel (59). Complete 4H, as well as incomplete junctions, were formed by annealing equal molar amounts of the appropriate gel-purified oligonucleotides in annealing buffer (10 mM Tris, pH 7.2, 50 mM NaCl, 10 mM $MgCl_2$) and treating the products as previously described (35). Prior to annealing, if needed, one of the oligonucleotides was 5′-end-labeled using T4 kinase. Stable formation of complete 4H (as well as incomplete junctions) was demonstrated by gel electrophoresis (data not shown). Nomenclature for four-way DNA junctions are defined in Lilley *et al.* (22) to be HHHH or 4H, representing four helixes converging to a central point. However, in addition to using complete 4H constructs in this study we also used incomplete junction constructs, i.e. those lacking one or more legs. The standardized nomenclature for our incomplete junction molecules is as follows: incomplete junction formed by annealing only oligonucleotides 3 and 4 (legs 3:4), HS_{14}/S_{20}; the incomplete junction formed by annealing only oligonucleotides 1, 3 and 4 (legs 1:3:4), $2HS_{14}/S_{16}$. This nomenclature indicates that there are either one or two converging DNA helixes with two single-strand extensions, the length of which are numerically indicated as subscripts (see Fig. 3 for an illustration).

Production and purification of proteins

HMG-1 and H1 proteins were purified from calf thymus and fractionated using a polybuffer BPE-94 column as described in detail by Adachi *et al.* (60). Recombinant human HMG-I(Y) was prepared as previously described by Nissen *et al.* (61).

Competition electrophoretic mobility shift assay

Electrophoretic mobility shift assays (EMSA) were performed by described procedures (59) by incubating 8 or 17 fmol labeled 4H with a target protein at room temperature in a total volume of 20 µl protein binding buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 25 mM NaCl, 0.28 µg sheared salmon sperm DNA, 2.8 µg BSA). Competitions were performed by adding unlabeled competitor DNA (either 4H or incomplete junctions) or competitor proteins to the reaction and incubating the combined mixture for an additional 10 min prior to electrophoresis. Glycerol was then added to 2% final concentration and the samples loaded onto a polyacrylamide gel (1.5 mm thick, 15 × 15 cm and comprised of 0.5× TBE, 4 or 6.5% polyacrylamide at 29:1 bisacrylamide). Gels were pre-run for at least 1 h at 10 V/cm and the electrode buffer replaced before loading samples. Samples were electrophoresed at 10 V/cm for 3–6 h at 4°C, following which the gels were dried on Whatman filter paper and exposed to either Amersham hyperfilm or to a Molecular Dynamics (Sunnyvale, CA) phosphorimager screen. Visual reproductions of the gels were created using a Textronix Phaser 440 printer. Quantification of band densities was performed using ImageQuant Peakfinder (Molecular Dynamics Corp., Sunnyvale, CA) to locate the bands and then the individual peaks were deconvoluted and analyzed using Peakfit (Jandel Scientific Corp., Corte Madera, CA). Further analysis, manipulations or plots were performed using Excel (Microsoft), Sigmaplot (Jandel Scientific) or Enzfitter (Niles & Associates, Berkeley, CA) software.

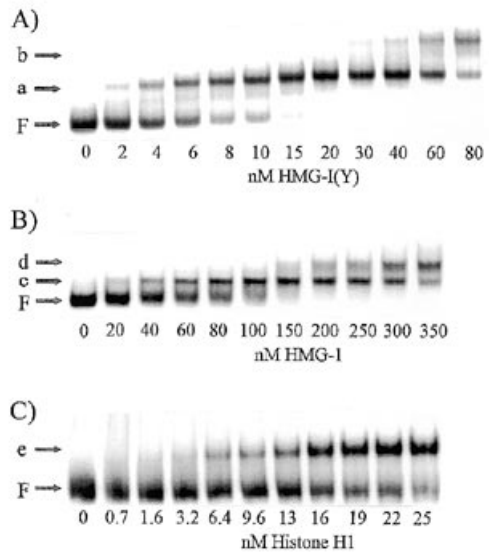

Figure 1. EMSA using 8 fmol labeled 4H titrated with increasing concentrations of (**A**) HMG-I(Y), (**B**) HMG-1 and (**C**) H1 proteins. Free migrating 4H is labeled F and the protein concentrations (in nM) are indicated at the bottom of each lane. (A) HMG-I(Y) formed two distinct complexes with 4H, labeled a and b. (B) HMG-1 also formed two distinct complexes with 4H, labeled c and d. (C) H1 only formed one complex with 4H, labeled e.

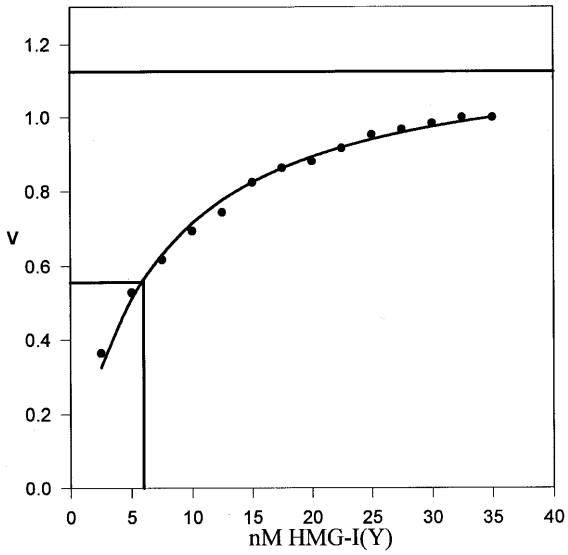

Figure 2. Binding curve of HMG-I(Y) on 4H. The relative band intensities of the first complex a from Figure 1A (and other gels not shown) were analyzed and plotted as described in Materials and Methods. The average number of protein molecules bound to a single 4H molecule (v) is indicated on the ordinate and molar concentration of HMG-I(Y) on the abscissa. The least squares fit curve (line) through the data points (dots) forms a rectangular hyperbola which indicates that binding of HMG-I(Y) to 4H is not cooperative and the K_d at $\frac{1}{2}v_{max}$ HMG-I(Y) is ~6.5 nM.

Determination of binding constants

Disassociation constants for HMG-I(Y), HMG-1 and H1 were determined by evaluating the relative band densities from EMSAs produced by titrating 4H with HMG-I(Y), HMG-1 and H1 (Fig. 1A–C). Prior to electrophoresis the reaction mixtures were incubated sufficiently long to reach equilibrium, so standard equilibrium equations describing ligand–substrate complex formation were used (62,63). The equilibrium equation used to describe the first, or tightest binding, DNA–protein complex (bands a, c and e in Fig. 1) is $P + A \leftrightarrows PA$, where P is the substrate (4H), A is the ligand (protein) and PA is the substrate–ligand complex. The average number of bound ligand molecules per substrate molecule is v, where $v = [PA]/[P] + [PA]$ and is related to the dissociation constant K_d by the equation $v = ([A]/K_d)/(1+[A]/K_d)$. K_d determinations were confirmed by plotting v versus $[A]$, which produces a rectangular hyperbola that approaches v_{max} (total ligands bound per substrate molecule). Extrapolation through the curve at $\frac{1}{2}v_{max}$ gives the K_d for the test protein (62,63).

Hydroxyl radical footprinting of HMG-I(Y) on 4H

Hydroxyl radical footprinting of HMG-I(Y) on 4H was carried out following published protocols (64). Each 100 µl reaction mixture containing 0.068 nM labeled 4H and enough HMG-I(Y) to form either one or two DNA–protein complexes was incubated at room temperature for 10 min prior to sequential addition of 4 µl each of: (i) a freshly prepared mixture of 0.75 mM Fe(II) and 1.5 mM EDTA; (ii) 37 mM ascorbate; (iii) 1.25% hydrogen peroxide. The complete reaction mixture was incubated at room temperature for exactly 2 min and stopped by addition of glycerol to 5%. To demonstrate the formation of a 4H–HMG-I(Y) complex, 5 µl of the mixture was loaded onto an EMSA gel (previously described) and electrophoresed. The DNA in the remaining reaction mixture was extracted with phenol:chloroform, dried, dissolved in formamide loading buffer, boiled for 5 min and then equal counts were loaded onto a 15% polyacrylamide sequencing type gel. The gels were electrophoresed at 18 000 V for 1.5 h, fixed in 8% acetic acid, 8% methanol, dried on Whatman paper and exposed to Amersham hyperfilm or a Molecular Dynamics phosphorimager screen.

RESULTS

Determination of the binding affinities of HMG-I(Y), HMG-1 and H1 for 4H

Specific protein interactions with 4H were studied using an EMSA. Radiolabeled 4H was titrated with increasing amounts of HMG-I(Y), HMG-1 and H1 proteins (Fig. 1A–C) to demonstrate that all three proteins specifically bind to 4H under identical conditions, but with different affinities. It is apparent from Figure 1A and B that HMG-I(Y) and HMG-1 are capable of forming two distinct complexes with 4H [labeled a and b for HMG-I(Y) and c and d for HMG-1], whereas H1 forms only one complex (Fig. 1C, band e). The composition of the putative 4H–HMG-I(Y) complexes was confirmed in a reciprocal control experiment in which unlabeled 4H was titrated with increasing amounts of *in vitro* ^{32}P-radiolabeled HMG-I(Y) protein and again, as expected, formation of the same two DNA–protein complexes was observed (data not shown). In each of the above titrations (Fig. 1A–C) the band densities of the free DNA as well as the first retarded DNA–protein complexes (i.e. bands a, c and e) were quantitatively analyzed as described in Materials and Methods. Using one-site binding parameters as descriptors for formation of the first retarded complex for HMG-I(Y), HMG-1 and H1 with 4H, the

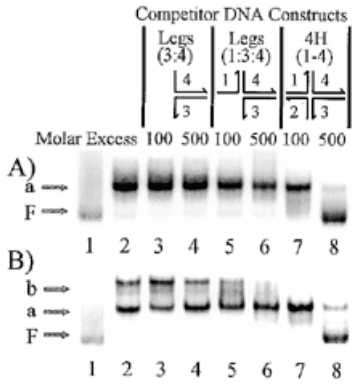

Figure 3. Competition EMSAs between 4H–HMG-I(Y) complexes and competitor DNAs were performed by incubating 17 fmol labeled 4H with (**A**) 10 or (**B**) 70 nM HMG-I(Y). Free radiolabeled 4H is labeled F and the first and second 4H–HMG-I(Y) complexes are labeled a and b respectively. Unlabeled competitor DNA, whether incomplete junction HS_{14}/S_{20} (legs 3:4), $2HS_{14}/S_{16}$ (legs 1:3:4) or complete 4H, were formed by annealing the appropriate legs together and added to the reaction mixtures at 100- and 500-fold molar excess, as indicated above each lane, prior to gel electrophoresis. The arrows on the 4H structure indicate the 3′-end of the oligonucleotides.

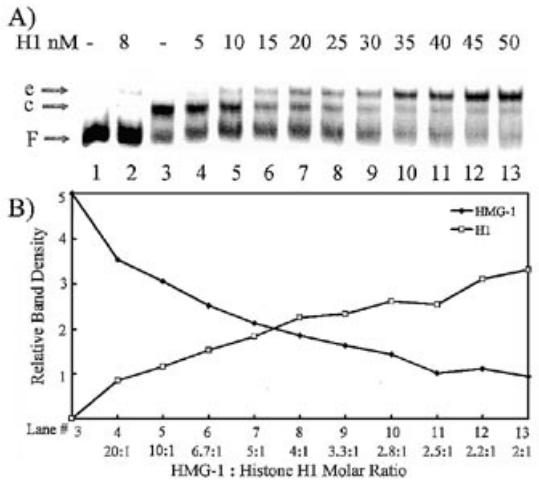

Figure 4. (**A**) Protein competition EMSA in which 17 fmol labeled 4H was preincubated with 80 nM HMG-1 (lanes 3–13) and titrated with increasing amounts of H1 (lanes 4–13) at the concentrations indicated above each lane. F indicates free migrating 4H, c indicates the position of the first 4H–HMG-1 complex and e indicates the position of the 4H–H1 complex band. (**B**) The relative band densities of bands c and e from (A) (lanes 3–14) were plotted versus molar HMG-1:H1 ratios. The labeled 4H is equally partitioned between the two proteins when the HMG-1:H1 molar ratio is ~5:1.

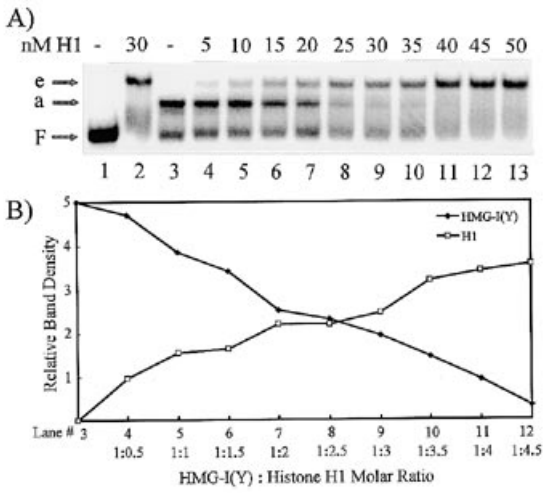

Figure 5. (**A**) Protein competition EMSA in which 17 fmol labeled 4H was preincubated with 10 nM HMG-I(Y) (lanes 3–13) and increasing amounts of H1 were added to the reaction mixtures (lanes 4–13) at the concentrations indicated above each lane (nM). F indicates the location of the free migrating 4H band, a indicates the location of the first 4H–HMG-I(Y) complex and e indicates the position of the 4H–H1 complex band. (**B**) The relative densities of bands a and e from (A) (lanes 3–12) were plotted versus HMG-I(Y):H1 molar ratios. The labeled 4H is equally partitioned between the two proteins when the HMG-I(Y):H1 ratio is ~1:2.5.

binding affinities for these proteins were determined to be ~6.5 nM for HMG-I(Y), ~16 nM for HMG-1 and ~80 nM for H1. The graph shown in Figure 2 clearly demonstrates that HMG-I(Y) does not bind cooperatively to 4H at its high affinity site, since the curve is not sigmoidal. In addition, this plot indicates that the K_d for HMG-I(Y) binding to 4H is ~ 6.5 nM, as determined by extrapolation through the point on the curve at $\frac{1}{2}v_{max}$. The K_d values for HMG-1 and H1 binding to their high affinity site on 4H were determined in a similar manner. These binding results indicate, as will be demonstrated below, that in competitive binding experiments HMG-I(Y) should have a dominant advantage over the other two proteins for binding to either 4H or to other DNA substrates with similar structural features.

Successive complex formation with increasing protein concentration, as demonstrated in Figure 1A and B, indicates that both HMG-I(Y) and HMG-1 have both a high affinity site (band a or c) and a lower affinity site (band b or d). For technical reasons based on the limitations of EMSA we were unable to quantitatively determine the binding affinity of HMG-I(Y) and HMG-1 to their lower affinity sites (65). Nevertheless, from a careful qualitative evaluation of the EMSA results shown in Figure 1A and B and other EMSAs not shown, the affinities of these proteins for their low affinity sites appear to be ~80 nM for HMG-I(Y) and ~300 nM for HMG-1. Regardless of the actual values, it is readily apparent from these electrophoretic results that HMG-I(Y) is binding much more tightly to 4H in its first complex than in its second complex. The same is true of HMG-1 and its two DNA–protein complexes.

The substrate titration results shown in Figure 1A demonstrate that HMG-I(Y) forms two specific retarded DNA–protein complexes with 4H. Since under identical conditions HMG-I(Y) protein does not physically associate with itself (unpublished observations), these results suggest that there is more than one binding site for the protein on 4H. Importantly, for both theoretical considerations and for the competition analyses discussed below, these quantitative ligand binding analyses clearly demonstrate that the first 4H–HMG-I(Y) complex observed during electrophoretic mobility shift assays (i.e. complex a in Fig. 1A) contains only one tightly bound HMG-I(Y) molecule rather than a complex of protein molecules bound to a single DNA substrate. These results are also consistent with the observation that HMG-I(Y) does not bind cooperatively to 4H (Fig. 2). Similar quantitative analyses indicate that complex c

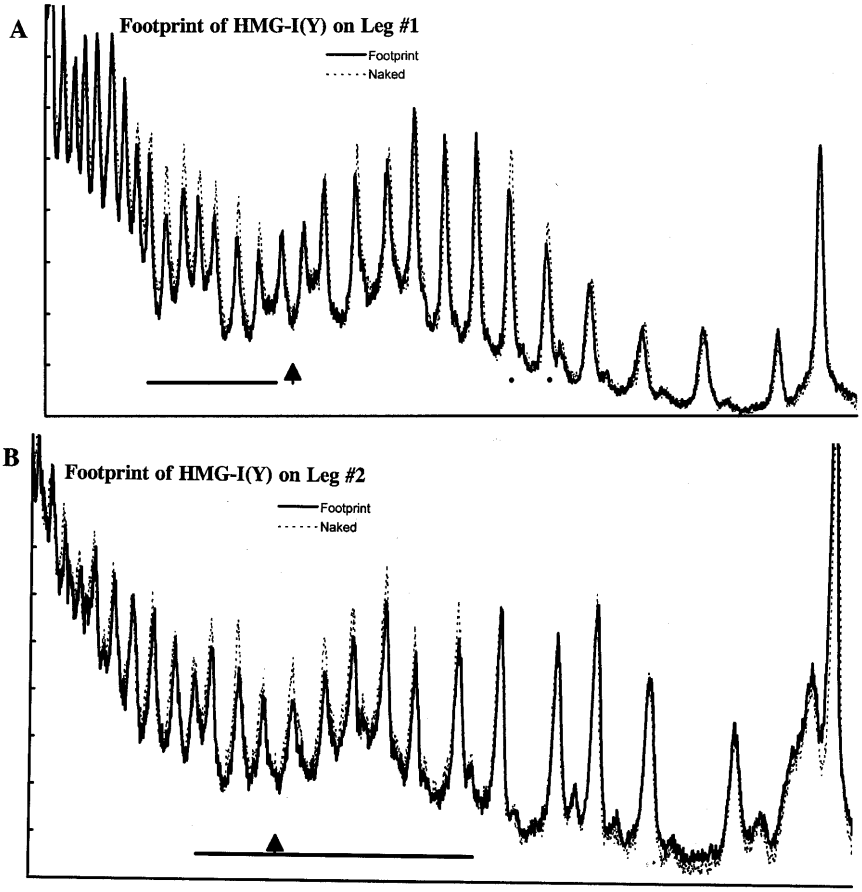

Figure 6. Hydroxyl radical cleavage data of 4H while in a 1:1 DNA–protein complex with HMG-I(Y). Densitometry scans of cleaved naked (dotted) 4H and complexed (solid) 4H–HMG-I(Y) on legs 1 (**A**) and 2 (**B**) are overlaid to demonstrate the difference in peak areas. The crossover of 4H is indicated by an arrow. The regions of greatest protection are indicated with a bar and the sites with minor protection are indicated with dots. The center of 4H is indicated by an arrow.

shown in Figure 1B contains a single HMG-1 molecule and complex e shown in Figure 1C contains a single H1 molecule (data not shown).

Specificity of HMG-I(Y) for 4H

Competition experiments were performed to determine the binding specificity of HMG-I(Y) to 4H. In these experiments HMG-I(Y) was incubated with labeled 4H and then titrated with increasing amounts of various unlabeled competitor DNAs. The competitor DNA molecules were either complete 4H or incomplete constructs, i.e. those lacking one or more legs (Fig. 3). In the first competition series (Fig. 3A) 20 nM HMG-I(Y) was incubated with 4H to form only the first high affinity 4H–HMG-I(Y) complex (a), which was subsequently competed with either a 100- or 500-fold molar excess of unlabeled competitor DNA. These results clearly show that the 4H–HMG-I(Y) complex (a) is not effectively competed with either of the incomplete junctions HS_{14}/S_{20} (legs 3:4) or $2HS_{14}/S_{16}$ (legs 1:3:4) (Fig. 3A, lanes 2–6), however, it is effectively competed with 4H (Fig. 3A, lanes 7 and 8). Similar results were obtained when incomplete junctions containing other combinations of legs were used in the competition assays (data not shown). These competition EMSAs demonstrate that high affinity binding of HMG-I(Y) to 4H requires an intact crossover-containing structure and also show that the HMG-I(Y) protein does not bind tightly to either the single-stranded regions or the elbows of the incomplete junction molecules.

A second competition series (Fig. 3B) was performed exactly like the first except that in this case a much higher concentration of HMG-I(Y) (70 nM) was initially incubated with labeled 4H to form both the high affinity (a) and the lower affinity (b) 4H–HMG-I(Y) complexes. This competition series demonstrates that the second 4H–HMG-I(Y) complex (b) is competed slightly with the incomplete junction HS_{14}/S_{20} (legs 3:4) and to a greater extent with the incomplete junction $2HS_{14}/S_{16}$ (legs 1:3:4). This result indicates that incomplete junctions possess some structural component that is weakly recognized by HMG-I(Y) but that this binding is not strong enough to dissociate the tightly bound protein from the first complex. The oligonucleotides used to form the incomplete 4Hs used in these competition assays contain a 5 bp stretch of A·T residues, the minimal length of duplex DNA required for specific binding of the HMG-I(Y) protein (39–44). However, the binding of HMG-I(Y) to this stretch of A·T residues in the duplex of legs 3:4 is relatively weak since neither of the incomplete junction constructs effectively competed with the first 4H–HMG-I(Y) complex, as demonstrated in both Figure 3A (lanes 2–6) and Figure 3B (lanes 2–6). Only the complete 4H construct effectively competed with the high affinity 4H–HMG-I(Y) complex (a) (Fig. 3B, lanes 7 and 8).

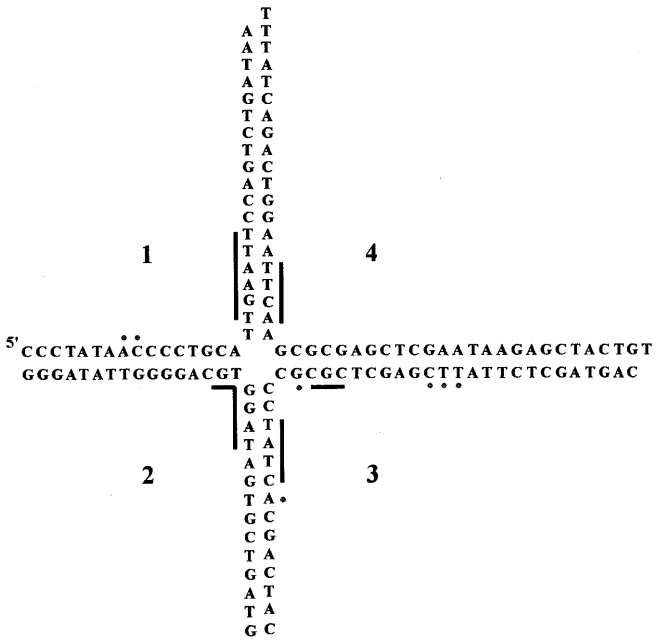

Figure 7. A composite figure showing the sequence and regions of the 4H that HMG-I(Y) protects from hydroxyl radical cleavage while in a 1:1 DNA–protein complex. Bars represent the regions of major protection and dots indicate areas of minor protection.

These titration experiments clearly demonstrate that HMG-I(Y) is more tightly associated with the first (a) complex than with the second (b) complex; they also indicate that the protein present in the b complex is actually binding to the duplex leg formed by legs 3 and 4 rather than to some other part of the incomplete structure. Furthermore, the first 4H–HMG-I(Y) complex (a) is only effectively competed by the intact four-way junction, suggesting that the HMG-I(Y) protein in this high affinity complex is associated with the junction or crossover region of 4H. As will be seen below, footprinting experiments with the HMG-I(Y) protein on 4H confirm these interpretations.

Strategy of the competition experiments

To investigate a possible hierarchy of binding of HMG-I(Y), HMG-1 and H1 we performed a number of competition EMSAs by first incubating labeled 4H with one protein and then titrating the preformed 4H–protein complex with a second protein. By design, in all of the competition experiments protein concentrations were such that only competition for binding to the highest affinity site for each protein was monitored. Two possible results were anticipated under these binding conditions: (i) direct competition between the titrated protein and the prebound protein for 4H binding, as evidenced by depletion of the original preformed DNA–protein complex band and concomitant appearance of a new complex band comprised of only the added competitor protein and 4H; (ii) simultaneous binding of the two proteins to the same 4H as evidenced by depletion of the preformed complex band and concomitant appearance of a new band representing a ternary complex comprised of 4H and both proteins.

Competition between HMG-1 and H1

The results of competition EMSAs between HMG-1 and H1 demonstrated that: (i) H1 and HMG-1 cannot bind simultaneously on the same 4H substrate; (ii) H1 can effectively displace HMG-1 from 4H (Fig. 4). The competition was conducted by incubating labeled 4H with only enough HMG-1 to form the first complex (c) and then titrating with increasing amounts of H1 (Fig. 4A). This gel clearly shows that during titration the intensity of the 4H–HMG-1 complex band (c) decreases concomitantly with an increase in intensity of the band corresponding to the 4H–H1 complex (e). Experiments using higher initial concentrations of bound HMG-1 (e.g. 100 nM) were also conducted and produced similar results (data not shown), however, slightly higher amounts of H1 were necessary to completely compete the HMG-1 from the preformed complex, which is expected under the given equilibrium conditions (62,63,65). These results indicate that H1 and HMG-1 compete with each other for the same binding site on 4H and that H1 easily out-competes HMG-1 for this site. Plotting the relative band intensities of the 4H–HMG-1 complex (c) and the 4H–H1 complex (e) from Figure 4A as a function of HMG-1:H1 molar ratio demonstrates that it takes an ~5 molar excess of HMG-1 over H1 to compete equally for the 4H substrate (Fig. 4B). We also conclude from these experiments and the fact that the K_d of H1 for 4H is ~16 nM, whereas that of HMG-1 is ~80 nM (Fig. 1), that binding of the H1 and HMG-1 proteins to 4H is mutually exclusive. Furthermore, it is evident from these results that H1 is clearly dominant over HMG-1 in *in vitro* competition assays using 4H as substrate and they suggest that this may likewise be the case for binding of these proteins to DNA crossover structures existing *in vivo*.

Competition between HMG-I(Y) and H1

Competition EMSAs between HMG-I(Y) and H1 demonstrated that HMG-I(Y) is very effective at competing with H1 for binding to 4H. Under equilibrium conditions 10 nM HMG-I(Y) was incubated with labeled 4H (i.e. enough protein to form only the tightest binding complex (a) and increasing amounts of H1 were then added (Fig. 5A). The result of this competition clearly shows disappearance of 4H–HMG-I(Y) band a with a concomitant appearance of 4H–H1 band e, indicating that H1 directly competes with HMG-I(Y) for the same site on 4H and that binding to this site by the two proteins is mutually exclusive. Other titrations using higher HMG-I(Y) concentrations (20 nM) were also performed and similar results were obtained (data not shown), however, somewhat greater amounts of H1 were needed to completely compete with the preformed complex of HMG-I(Y), a result that is expected under equilibrium conditions (62,63,65). Again, as shown in Figure 5B, we plotted the relative band densities versus the molar ratio of protein concentrations. This graph clearly indicates that H1 must be in at least a 2.5 molar excess over HMG-I(Y) to compete equally for substrate binding, a result consistent with the relative K_d values of these two proteins for 4H substrates (Fig. 1). It is therefore evident from these results that HMG-I(Y) is able to out-compete H1 for binding to 4H *in vitro* and, most likely, also *in vivo*.

Competition between HMG-I(Y) and HMG-1

Competition EMSAs were conducted using HMG-I(Y) and HMG-1 proteins. However, because the 4H–HMG-I(Y) and 4H–HMG-1

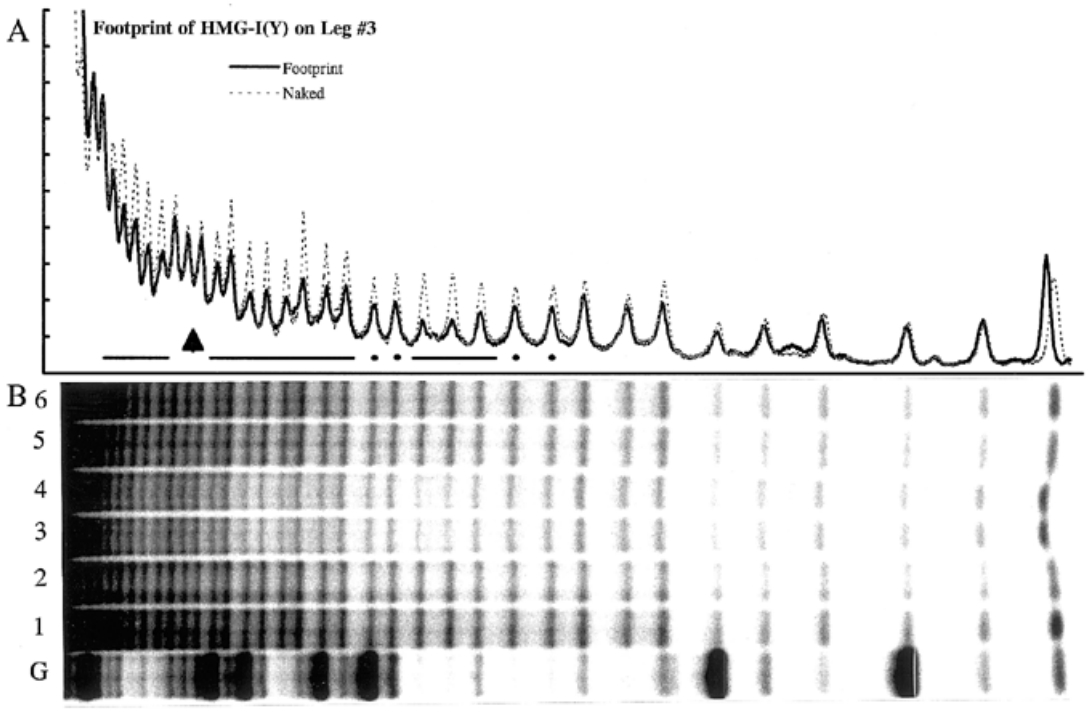

Figure 8. Hydroxyl radical footprint data of HMG-I(Y) on 4H in a 1:2 DNA–protein complex. (**A**) A densitometry scan from lanes 2 (dotted is naked DNA) and 3 [solid is complexed 4H–HMG-I(Y)] indicating relative band densities of the footprinting gel (B). (**B**) The autoradiogram showing the footprint of HMG-I(Y) on leg 3 of 4H (lanes 3 and 4). Lanes 1, 2, 5 and 6 are control lanes which were treated in the absence of HMG-I(Y) (naked 4H). The lowest lane G is a Maxam–Gilbert G lane used as a marker. Electrophoresis is from left to right. Footprinted regions were determined by calculating the difference in peak areas between the footprinted and naked DNA, the regions of greatest protection are indicated with bars and the areas of minor protection are indicated with dots. The crossover of 4H is indicated by an arrow.

complexes have nearly identical mobilities under the several different gel electrophoretic conditions examined, we were unable to determine the precise HMG-I(Y):HMG-1 molar ratios contained in the shifted band, rendering the gel systems employed unsuitable for quantitative competition comparisons. Nevertheless, we did not observe any ternary complex formation in any of the competition titrations, indicating that HMG-I(Y) also competes with HMG-1 for binding to the high affinity site on 4H (data not shown).

Hydroxyl radical footprinting of HMG-I(Y) on 4H

Hydroxyl radical DNA cleavage experiments were employed to determine the placement of binding of HMG-I(Y) protein on 4H at base pair resolution. Our footprinting of HMG-I(Y) on 4H is the first of its kind, since, to our knowledge, no other mammalian HMG proteins have been footprinted on 4H. The results shown in Figures 6–9 indicate that HMG-I(Y) binds most strongly to 4H near the junction of the four oligonucleotide strands. Figures 6 and 7 show the results of hydroxyl radical footprinting of a 1:1 4H–HMG-I(Y) complex (i.e. complex a in Fig. 1), whereas Figures 8 and 9 depict the results of footprinting a 1:2 4H–HMG-I(Y) complex (i.e. complex b in Fig. 1).

The results of a typical hydroxyl radical footprint of HMG-I(Y) on 4H can be seen in the autoradiogram shown in Figure 8B, where 4H containing a single labeled oligonucleotide (i.e. 3) was incubated with enough HMG-I(Y) protein to form both high and low affinity DNA–protein complexes (i.e. complex b in Fig. 1). The band intensities from the hydroxyl radical cleavage reactions of naked 4H (lane 2) and protein-complexed 4H–HMG-I(Y) (lane 3) were quantitatively analyzed by densitometry, normalized and the resulting scans overlaid to produce the composite results shown in Figure 8A. The bars indicate the regions of greatest protection of 4H by HMG-I(Y) from cleavage and the dots represent less protected regions. All four legs of the oligonucleotide strands comprising the 4H structure were independently radiolabeled, formed into DNA–protein complexes, subjected to hydroxyl radical footprinting, analyzed and the composite results of numerous such experiments are depicted in Figure 9.

Hydroxyl radical footprinting was also carried out on only the high affinity 4H–HMG-I(Y) complex (i.e. a in Fig. 1A). As previously described, 4H was complexed with HMG-I(Y) at a 1:1 ratio and subjected to hydroxyl radical cleavage. The band intensities from the hydroxyl radical cleavage reactions of naked 4H and complexed 4H–HMG-I(Y) were quantitatively analyzed by densitometry and the resulting scans overlaid to produce the composite results shown in Figure 6. All four legs were likewise analyzed and the composite results of numerous such experiments are depicted in Figure 7.

Footprinting of the 1:1 and 1:2 (DNA–protein) complexes of 4H–HMG-I(Y) indicate that HMG-I(Y) binds preferentially to the crossover at the center of 4H. However, the footprint of the 1:2 complex differs somewhat from that of the 1:1 complex, since in the 1:2 complex there is greater protection of the 4H legs emanating out from the central area of the crossover. In addition, in the 1:2 4H–HMG-I(Y) complex there is an increase in protection of short (5 bp) runs of A·T sequences located on the duplex legs formed by oligonucleotides 1:2 and 3:4 (Fig. 9) due

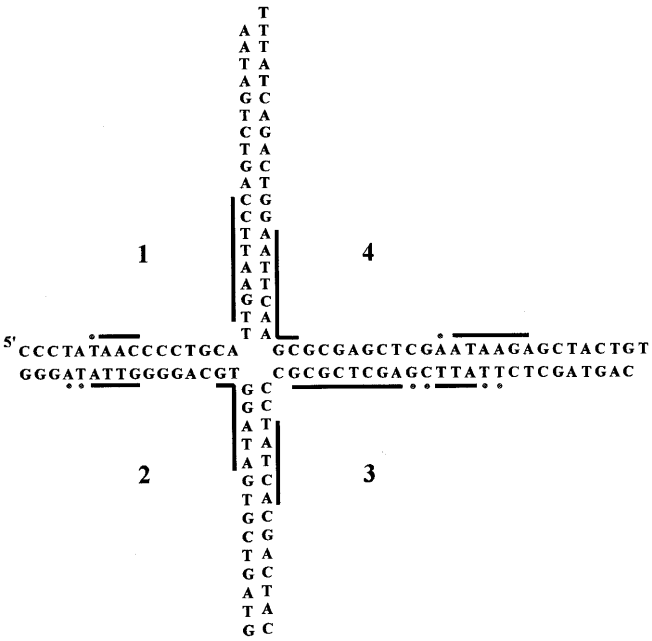

Figure 9. A composite figure showing the sequence and regions of 4H that HMG-I(Y) protects from hydroxyl radical cleavage while in a 1:2 DNA–protein complex. Bars represent the regions of greatest protection and dots indicate areas of minor protection.

to low affinity binding of the protein. These footprinting experiments unequivocally demonstrate that HMG-I(Y) binds most tightly to the center of the junction, which is relatively free of A·T residues, indicating that HMG-I(Y) recognizes the non-B-form structure, rather than the sequence, of the DNA in this region of the molecule. The results also show that HMG-I(Y) binds less tightly, but, as expected (42–44), to a region of duplex DNA containing short runs of A·T nucleotides located on the legs of 4H.

DISCUSSION

Four-way junction DNA was used as a substrate in our *in vitro* protein binding studies because it is thought to mimic the Holliday-type recombination crossover structures found *in vivo* (52,66–69) and also because it has been hypothesized to simulate the structure of the linker DNA strands near the entrance and exit points of nucleosomes (21,30,70). With these biological connections in mind, it is significant that the HMG-1/-2 proteins have recently been shown to participate in genetic recombination events during immunoglobulin gene rearrangements *in vitro* (71). Because HMG-I(Y) out-competes HMG-1 for binding to 4H it is also reasonable to expect that HMG-I(Y) may likewise facilitate genetic recombination *in vitro*. In addition, HMG proteins have recently been demonstrated to be required for integration of retroviral cDNAs into host cell DNA substrates *in vitro*, a process that in many respects is homologous to recombination events involving 4H structures. For example, HMG-1 has been shown to be required for efficient integration of avian sarcoma virus into host cell DNA *in vitro* (72) and, likewise, HMG-I(Y) has been demonstrated to be required for integration of HIV-1 cDNA into DNA *in vitro* (73). Although little information is currently available, in the light of our present findings it seems reasonable to suspect that H1 might likewise participate in genetic recombination and/or retroviral integration events.

As discussed above, the HMG-I(Y), HMG-1 and H1 proteins have all been demonstrated to specifically associate with nucleosomal chromatin *in vitro* and 4H has been suggested to structurally resemble linker DNA near the entrance and exit points of nucleosomes. Nevertheless, results from our quantitative binding studies for each of these individual proteins to 4H do not closely correlate with the previously reported binding affinities of these proteins to nucleosomal substrates. For example, we find that H1 binds to 4H with a K_d of ~16 nM, which is similar to the K_d value of ~18 nM reported by others for H1 binding to naked B-form DNA *in vitro* (14). In contrast, H1 has been reported to have a higher *in vitro* binding affinity for both reconstituted *Xenopus* 5S rDNA mononucleosomes (K_d ~2 nM) (10) and dinucleosomes (K_d ~7.4 nM) (14). In the case of HMG-1 we find that it binds to 4H with a K_d of ~80 nM, whereas HMG-1 has been reported to have a much lower affinity for reconstituted 5S rDNA dinucleosomes (K_d ~300 nM) (14). In marked contrast to histone H1, HMG-I(Y) protein binds more tightly to 4H (K_d ~6.5 nM) than to random sequence nucleosome core particles (K_d ~50 nM) (46). Thus our data suggest that *in vitro* the binding of HMG-I(Y), HMG-1 and H1 to 4H probably more closely relates to *in vivo* biological events such as genetic recombination or retroviral integration than to binding of these proteins to linker DNA near the entrance and exit points of nucleosomes.

Our protein binding competition studies are also consistent with a previous report indicating that HMG-I(Y) has the ability to displace H1 from duplex linear B-form DNA containing A·T-rich MAR/SAR sequences (74,75). In combination, these findings indicate that HMG-I(Y) is able to out-compete H1 for binding to different substrates, whether they are the A·T-rich, B-form DNA sequences found in MAR/SAR regions or the kinked, distorted DNA structures found at the crossover of 4H. In both cases HMG-I(Y) has the capacity to effectively displace H1 from such substrates. These observations are significant because DNA in the living cell is believed capable of assuming many different configurations and it is likely that, regardless of its biological form, HMG-I(Y) will have the ability *in vivo* to out-compete, or displace, H1 from such DNA structures.

Finally, it should also be noted that our hydroxyl radical footprints of the high affinity binding site of HMG-I(Y) on 4H are consistent with a previously reported footprint of a prokaryotic HMG-1-like protein, HU, on 4H. In this instance HU, which associates as a dimer, binds on opposite sides of the crossover region of 4H (76). Both our results and those with HU protein (76) are consistent with the suggestion by Lilley (30,52) that 4H *in vitro* has a 2-fold symmetrical structure that provides independent binding sites, located on opposite sides of the junction, for structure-recognizing proteins.

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