A Small Region in HMG I(Y) Is Critical for Cooperation with NF-κB on DNA*

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The high mobility group HMG I(Y) protein has been reported to promote the expression of several NF-κB-dependent genes by enhancing the binding of NF-κB to DNA. The molecular origins of cooperativity in the binding of NF-κB and HMG I(Y) to DNA are not well understood. Here we have examined the determinants of specificity in the binding of HMG I(Y), both alone and in cooperation with NF-κB, to two different DNA elements, PRDIV from the interferon-β enhancer and IgκB from the immunoglobulin k light chain enhancer. Of particular interest was the influence of a flanking AT-rich sequence on binding by HMG I(Y). Utilizing yeast one-hybrid screening assays together with alanine-scanning mutagenesis, we have identified mutations of residues in HMG I(Y) that decrease cooperative binding of NF-κB to PRDIV and IgκB sites. These same mutations similarly decreased the binding of HMG I(Y) alone to DNA, and paradoxically, decreased the strength of protein-protein interactions between HMG I(Y) and NF-κB. Of the three tandemly repeated basic regions that represent putative DNA-binding motifs in HMG I(Y), the residues within the second repeat are most important for recognition of core NF-κB sites, whereas the second and third repeats both appear to be involved in binding to sites that are flanked by AT-rich sequences. Overall, the second repeat of HMG I(Y) is primarily responsible for the stimulatory effect of this protein on the binding of NF-κB to PRDIV and IgκB elements.

HMG I(Y) protein belongs to the family of high mobility group (HMG) non-histone chromosomal proteins that binds preferentially in the minor groove of AT-rich sequences in DNA (1). The expression of HMG I(Y) is normally very low in adult tissues, but is up-regulated in the rapidly proliferating cells found in embryonic and neoplastic tissues, leading to the suggestion that the HMG I(Y) gene might be directly involved in development and neoplasia (2). HMG I(Y) has also been implicated as a structural component involved in the condensation of AT-rich regions of mammalian chromosomes (3), and has been shown to be a host protein required for integration by HIV-1 preintegration complexes in vitro (4).

HMGI(Y) and HMG Y are isoforms produced by alternative splicing of a single functional pre-mRNA, with 11 internal amino acids present in HMGI but deleted in HMG Y (3); the function of this 11-amino acid stretch is not known, hence the two isoforms are designated interchangeably as HMG I(Y). HMGI(Y) and HMG I-C, the other member of HMG I(Y) family, are both small (∼10 kDa) proteins, which share three highly conserved basic regions arranged in tandem, in addition to an acidic COOH-terminal domain. The basic regions are essential for DNA binding, whereas the COOH-terminal domain facilitates sequence-specific interaction with DNA (5). The consensus basic repeats possess a unique structure referred to as an “AT-hook,” which binds deeply into the minor groove of the AT-rich DNA recognition sites (6, 7).

HMG I family proteins can serve as either positive or negative accessory factors for gene transcription regulated by a wide range of transcription factors, including those of the Rel, bZIP, ETS, and POU families (8–11). HMGI(Y) proteins do not appear to function independently as transcriptional activators or repressors (12), even though they possess an acidic domain reminiscent of that often found in many transcriptional activator proteins (13). Instead, they bind to certain AT-rich sequences within or flanking transcription factor recognition sites, either enhancing (10, 11) or competing with (14, 15) the binding of these transcription factors to adjacent or overlapping DNA sites.

Among the loci reported to be regulated by HMG I family proteins, the most extensively characterized is the human interferon-β (IFN-β) gene (16), which is transcriptionally activated upon viral infection. The gene enhancer consists of a negative regulatory domain NRDI (17), as well as four adjacent positive regulatory domains (PRDs) organized in the order: PRDIV, PRDI-PRDIV, and PRDIV; these are recognized, respectively, by the transcription factors NF-κB (18), IFN-regulatory factor 1 (19), and activating transcription factor 2 (ATF-2/c-Jun) (20). HMGI(Y) binds specifically to one site within NRDI, one site in PRDIV, and two sites in PRDIV (5). Rather than four HMGI(Y) molecules individually contacting these four different sites, recent evidence indicate that two HMGI(Y) molecules bind the enhancer, with each protein molecule occupying two closely juxtaposed sites. Specifically, the second and third repeats of HMGI(Y) simultaneously bind PRDIV and NRDI, whereas the second and first repeats bind the two sites within PRDIV (5, 21). In both cases, the tandem HMGI(Y)-binding sites are separated by one full helical turn, and the binding interaction is highly cooperative. All four HMGI(Y) sites within the enhancer are essential for optimal virus induction of the IFN-β gene. The binding of HMGI(Y) to the minor groove of PRDIV stimulates NF-κB binding to the major groove of the same site approximately 10-fold in vitro (11). Similarly, the affinity of ATF-2 for PRDIV increases about 4-fold upon coincubation with HMGI(Y) (8). Circular permutation and

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§ The abbreviations used are: HMG, high mobility group; IFN, interferon; PRD, positive regulatory domain; ATF, activating transcription factor; RHR, Rel homology region; GST, glutathione S-transferase; IRE, interferon response element; bp, base pair(s); EMSA, electrophoretic mobility shift assay; ONPG, o-nitrophenyl-β-D-galactopyranoside; BSA, bovine serum albumin; AD, activation domain.

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phasing analyses of IFN-β gene enhancer revealed that the intrinsic DNA bends in the enhancer can be reversed by the binding of NF-κB and ATF-2/c-Jun, and further modulated by HMG I(Y) (22). Taken together, these studies establish an important role for HMG I(Y) as an accessory protein which mediates the assembly and function of the IFN-β gene enhancer by promoting the cooperative binding of transcription factors to the enhancer (23, 24).

NF-κB is the prototype of the dimeric class of Rel proteins, members of which are characterized by the presence of an approximately 300-amino acid Rel homology region (RHR) that mediates protein-protein interactions, sequence-specific DNA recognition, and nuclear translocation (25). NF-κB plays a broad role in inducible and coordinate regulation of genes involved in inflammation, immune responses, cell growth and differentiation, and virus infection (26). Evidence suggests that NF-κB cooperates with HMG I(Y) at a variety of regulatory loci. In addition to the IFN-β gene, HMG I(Y) has also been reported to enhance NF-κB activity on several other promoters including E-selectin (27), interleukin-2/granulocyte-macrophage colony-stimulating factor (28), and chemokine MGS/GR0 α (29).

Despite the importance of the cooperative interactions between NF-κB and HMG I(Y), the mechanistic basis for this phenomenon is poorly understood. In principle, cooperative binding of NF-κB and HMG I(Y) to DNA could arise from either or both of two limiting scenarios: (i) the binding of one protein to its site might induce local structure changes in DNA that favor interaction of the other protein with its site; or (ii) NF-κB and HMG I(Y) might make favorable protein-protein contacts with each other. Consistent with the first scenario, HMG I(Y) can reduce the intrinsic DNA curvature of PRDII and enhance the reversal of PRDII bending by NF-κB (22). In keeping with the second scenario, HMG I(Y) has been shown to bind both NF-κB and ATF-2 in GST fusion protein binding assays (8–10), a procedure that can sometimes detect weak interactions that do not occur physiologically. Direct evidence has not been provided to show that NF-κB and HMG I(Y) can make energetically favorable contacts to each other on DNA recognition elements in vivo. Therefore, the contribution of direct protein-protein interactions to the cooperative binding of NF-κB and HMG I(Y) to DNA remains uncertain.

To investigate this issue and further analyze the NF-κB/HMG I(Y)/DNA interaction interface, while avoiding the potential complexity arising from the abundant endogenous HMG I(Y) and NF-κB proteins in mammalian cells, we have reconstituted the core NF-κB/HMG I DNA complex in yeast. Using this system, we investigated the DNA binding specificity of HMG I(Y) on two κB DNA motifs (the core NF-κB sites from PRDII and IgκB). We demonstrate that the discrimination of these two κB sites by HMG I(Y), in terms of both DNA recognition and cooperation with NF-κB, can be greatly enhanced by the presence of additional AT-rich sequences adjacent to the core κB DNA motifs. The use of yeast one-hybrid screening assays (30, 31) in combination with deletion and alanine-scanning mutagenesis (32) of HMG I enabled us to examine and compare mutations affecting NF-κB/HMG I cooperativity and HMG I DNA recognition on the PRDII or IgκB site, which either alone or flanked by NRDI as in the context of the entire interferon response element (IRE, including PRDI, PRDII, and NRDI). Intriguingly, we find that the mutations affecting NF-κB/HMG I cooperative DNA binding also affect HMG I DNA affinity to a similar extent. While the residues most important for core NF-κB site recognition fall within the second basic repeat of HMG I, the third repeat is identified as being crucial for HMG I/PRDII-NRDI interaction. Further in vitro characterization of the protein-protein interaction independent of DNA revealed that the same mutations in the second repeat abolished the interaction of HMG I with NF-κB p50 as well. Therefore, binding sites for the same region of HMG I exist in both NF-κB p50 protein and κB DNA motifs. These data imply that the second repeat of HMG I, through either binding to κB DNA motifs, or interacting with NF-κB p50, or both, contributes to the most cooperative binding of NF-κB and HMG I to DNA.

**Materials and Methods**

*Oligonucleotides.*—Blunt-ended 44-bp duplex probes 5'-GGAGAAGTTGTCGACAGTGGGAATCTTCCTCTGACTGAGAGGACG-3' containing the core NF-κB site (YY = AA) or an NF-κB site in which the sequence (5'-YXYXTTCTTG-3') was replaced (lowercase denotes single-stranded DNA overhang). The duplex EMSA probes containing PRDII-NRDI (YY = AA) or IgκB-NRDI (YY = CT) in the context of IRE were designed as 5'-GAAGTGAAGAAGGAACTTCCTCTGACTGAGAGGAGG-3'.

The corresponding yeast PRDII-NRDI (YY = AA) or IgκB-NRDI (YY = CT) reporter has been described previously (5'-AGCAGAAGGAACTTCCTGACTGAGAGGAGG-3'). A five-nt-regulator 5'-AGCAGAAGGAACTTCCTGACTGAGAGGAGG-3'.

*Yeast Plasmid Constructions.*—The yeast plasmid used to construct PRDII and IgκB reporters has been described previously (30). The RHRs of human NF-κB p50 (residues 41–353) and p65 (residues 1–290) were expressed under control of the GAL1 promoter on the yeast LEU2 selectable pCB195 and HIS3 selectable pAM423 plasmids, respectively. prf4–6NL-AD was constructed by insertion of the VP16 activation domain sequence into the EcoRI site of the yeast TRP1 selectable prf4–6NL plasmid. Full-length human HMG I was cloned into either pRF4–6NL-AD or prf4–6NL and the protein was expressed with or without an NH2-terminal fusion to VP16 activation domain. The human NF-κB RHRs and HMG I proteins were all expressed in yeast containing NH2-terminal hemaglutinin epitope tag and SV40 large T antigen nuclear localization sequence.

*β-Galactosidase Reporter Gene Assays.*—Yeast plasmids were transformed into the yeast strain FY250 (MATa, URA3-52, HIS3200, LEU231, TRP1463; gift of M. Ptashne, Harvard) by a simplified lithium acetate method. Yeast transformants grown on appropriate selection plates were inoculated into liquid selection media containing 2% raffinose as the carbon source to an OD of ~2. Protein expression was induced by the addition of galactose to a final concentration of 2%. After 4 h, β-galactosidase activity was quantified as described (30) with either α-nitrophenyl-β-D-galactopyranoside (ONPG, Sigma) or chloroform red-β-galactopyranoside (Calbiochem) substrates. Each assay shown in Figs. 2, 5, and 6 was performed in triplicate and the standard error was estimated to be within ±20%.

*Recombinant Proteins.*—The recombinant NF-κB p50 RHR (residues 1–366 or residues 1–366 with deletion of 2–39) was expressed and purified to homogeneity as described (33). The p65 RHR (residues 1–305) was the generous gift of R. Chopra and S. Harrison (Harvard University). Full-length HMG I and alanine mutant proteins were expressed as NH2-terminal or COOH-terminal His6 fusion proteins, and purified through Ni2+–NTA-agarose column (Qiagen); or as NH2-terminal GST fusion proteins, purified through glutathione-agarose column (Amersham Pharmacia Biotech).

*Gel Electrophoretic Mobility Shift Assays.*—Binding reactions with purified proteins and 5'-end-labeled duplex probe were performed in the binding buffer (10 mM Tris (pH 7.5), 50 mM NaCl, 5% glycerol, 5 mM MgCl2, 0.1% Nonidet P-40, 1 mM dithiobis) supplemented with various concentrations of nonspecific competitors (0.1–1 mg ml⁻¹ BSA, 5–50 μg ml⁻¹ of poly(dG-dC)). The mixture was incubated at room temperature for 15 min and analyzed by electrophoresis in a nondenaturing 5% polyacrylamide gel at 4°C in 0.5 × Tris borate-EDTA.

*DNase I Footprinting Assays.*—Duplex PRDII or IgκB oligonucleotide probes, 3'-32P-end-labeled on the coding strand, were incubated either

2. B. Cohen and R. Brent, unpublished results.
3. A. Mendelsohn and R. Brent, unpublished results.
4. R. Finley and R. Brent, unpublished results.
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Reconstitution of Core NF-κB/HMG I/DNA (PRDII or IgκB) in Yeast—To explore the mechanism of cooperativity between NF-κB and HMG I in vivo, we reconstituted the NF-κB-HMG I/DNA complex in yeast. Two κB motifs were chosen for study, PRDII and IgκB, each of these was examined in two contexts, either alone (core sites) or integrated into the IRE, wherein the NF-κB site is flanked by NRDI (PRDII-NRDI and IgκB-NRDI). These various permutations on κB motifs enabled us to compare protein binding and cooperativity as a function of sequence changes both within and flanking the core site. The two core NF-κB sites investigated here differ at only two positions in their AT-rich centers, 5'-GGGAAATTCC-3' (PRDII) versus 5'-GGGACCTTCCC-3' (IgκB). In x-ray crystallographic structures of NF-κB-DNA complexes (34–37), the protein mainly interacts with the GC-rich outer regions of the site, and all base contacts are made in the major groove. This leaves open the minor groove of the κB site, where HMG I(Y) is believed to interact mostly with the AT-rich center.

The RHRs of p50 and p65, and full-length HMG I fused to an NH2-terminal hemaglutinin epitope tag plus SV40 large T antigen nuclear localization sequence, were co-expressed in yeast and used to drive the expression of a lacZ reporter linked to a single core NF-κB site (PRDII or IgκB) or to the same site integrated into the IRE (PRDII-NRDI or IgκB-NRDI). Whereas the p50 RHR homodimer does activate transcription in yeast cells with reporters bearing core NF-κB sites (38) (data not shown), neither the p65 RHR homodimer alone nor HMG I alone gave detectable activation of lacZ reporter gene expression (data not shown). Co-expression of the p50 and p65 RHRs increases lacZ gene expression ~3-fold over that seen with the p50 RHR alone (data not shown), perhaps reflecting the higher DNA binding affinity of the p50:p65 heterodimer as compared with that of the p50:p50 homodimer.

In order to monitor the binding of HMG I alone to DNA in vivo, we fused the VP16 activation domain (AD) onto HMG I and measured the transcriptional activities of the resulting AD-HMG I chimera with lacZ reporter constructs. AD-HMG I is a reasonably strong activator of gene expression driven by the core PRDII site, but shows no activity on the core IgκB reporter (Fig. 1, compare lane 3 of the left- and right-hand panels), thus indicating that HMG I alone binds to core PRDII in vivo, but not to the core IgκB site, at least to the extent necessary to activate transcription. NF-κB and AD-HMG I together activate the PRDII reporter ~4-fold over the sum of the levels observed with NF-κB and AD-HMG I expressed separately (left panel, compare lane 4 to lanes 1 and 3). Even though AD-HMG I is unable by itself to activate the IgκB reporter, AD-HMG I and NF-κB together activate the IgκB reporter ~20-fold over the levels seen with NF-κB only (right panel, lane 4 versus 1 and 3). These synergistic effects in activation by AD-HMG I plus NF-κB could in principle result from mutual stabilization of DNA binding (binding cooperativity), or from multiplicity in activation domain contacts to the transcriptional machinery (transcriptional synergy) (39). To distinguish these possibilities, we removed the activation domain from AD-HMG I and tested its effects on reporter gene activation in combination with NF-κB (Fig. 1). HMG I lacking a fused activation domain stimulated NF-κB-driven expression of both PRDII and IgκB reporters by ~2–3-fold (compare lane 1 with 2 in each panel). These data indicate that the synergistic effects seen with NF-κB and HMG I result at least in part from cooperativity in their binding to DNA.

HMG I has been shown to bind PRDII and NRDI simultaneously through an intramolecularly cooperative interaction of the second and third basic repeats with the two juxtaposed sites in DNA (1:1 complex) (5). To compare the activation responses produced by this intramolecularly cooperative binding mode with those on core κB sites, we analyzed the transcriptional response driven by the PRDII-NRDI and IgκB-NRDI sites. As shown in Fig. 2, the NF-κB p50:p65 heterodimer alone barely activated PRDII-NRDI or IgκB-NRDI reporter gene expression (lanes 1), neither did HMG I with or without an attached AD (lanes 3). These findings were unexpected, because NF-κB gave significant levels of activation with both the core PRDII and IgκB sites, and AD-HMG I at least with core PRDII (Fig. 1). Although the reasons for these differences between core and NRDI-linked κB sites is not clear, one possibility is suggested by the fact that the insertion of NRDI into the reporter constructs moves the κB site 15 bp (1.5 turns of the DNA helix) further away from the transcription start site, as compared with the corresponding reporters containing core sites; these differences in helical phasing of the κB sites resulting from the presence or absence of NRDI may affect the presentation of the activation domain(s) to the transcription machinery. Such stereochemical effects have been found in certain cases to exert a powerful influence on transcriptional responses (40, 41). Notwithstanding these differences, NF-κB-driven expression of κB-NRDI reporters was stimulated by 3–4-fold by the presence of HMG I lacking an AD (lanes 2 versus 1); this magnitude is comparable to that seen with core κB reporters. Furthermore, co-expression of NF-κB and AD-HMG I led to activation of both PRDII-NRDI and IgκB-NRDI reporters to levels similar to those seen with core PRDII and IgκB reporters (lanes 4 versus 1 and 3), owing to a highly...
synergistic (~50-fold) activation response on these sites. In quantitative terms, the levels of synergy between NF-κB and AD-HMG I on the PRDII site are increased 10-fold by being linked to NRDI, as compared with a corresponding 2.5-fold increase for IgκB.

Specificity of HMG I Binding to DNA and Enhancement of NF-κB Binding in Vitro—To characterize directly the strength and specificity of HMG I binding to PRDII and IgκB sites in DNA, we performed EMSA using bacterially expressed HMG I-His6 fusion protein. Duplex oligonucleotide probes initially used in these experiments contained the core PRDII or IgκB sites flanked by non-AT-rich sequences. These probes differ in sequence at only the two positions in the center of the κB DNA motifs.

In preliminary EMSA experiments aimed at optimizing experimental conditions, we observed that the apparent \( K_d \) values of HMG I for both PRDII or IgκB sites were strongly influenced by the concentrations of nonspecific competitor protein and DNA (data not shown). Therefore, EMSA assays were carried out at several distinct concentrations of competitors. In the presence of 0.1 mg ml \(^{-1} \) BSA and 5 \( \mu \)g ml \(^{-1} \) poly(dG-dC) as nonspecific competitors, the COOH-terminal His6-tagged HMG I protein binds the core IgκB probe \textit{in vitro} with only a 2-fold higher \( K_d \) than on PRDII (80 versus 40 nM, respectively). To minimize nonspecific protein-DNA interaction with the flanking sequences, we decreased the length of the PRDII and IgκB probes from 44 to 20 bp. Although the affinities of HMG I for the shorter probes are decreased somewhat, the 2-fold difference in affinities for the core PRDII and IgκB sites remained unchanged (data not shown). To quantify the strength of nonspecific binding of HMG I under these conditions, EMSA assays were carried out with a 44-mer probe having 5 GC bp in place of the 5 AT bp in the core PRDII site, but otherwise identical to it. Surprisingly, HMG I binds this control probe with a \( K_d \) of 180 nM, which is only ~5-fold weaker than that for the core PRDII site (data not shown). This relatively low degree of sequence specificity contrasts with the higher specificity seen with sites that contain flanking AT-rich sequences (11, 42) (see "Discussion").

To identify directly the region in these core NF-κB probes to which HMG I binds, we carried out DNase I footprinting analysis. HMG I exhibits sufficient specificity in binding to the PRDII site to yield a pronounced footprint (Fig. 3A, left panel); however, changing the 2 bp in the middle of the PRDII site to those in IgκB leads to a significant deterioration of binding specificity, which can be inferred from both the weakness of the footprint in the IgκB site and from the general suppression of DNase I cleavage at positions outside the site. We also noted that the difference in \( K_d \) values for binding to the core κB sites measured by the DNase footprints is at least 4-fold, as compared with the 2-fold difference detected by EMSA. Most likely, this apparent discrepancy stems from the fact that the EMSA experiments give a composite readout of both nonspecific and specific DNA binding, whereas DNase footprints tend to reveal specific binding modes much more obviously than nonspecific. As the nonspecific \( K_d \) determined by EMSA is only 2.5-fold above the \( K_d \) for IgκB, it is expected that nonspecific binding modes contribute substantially to the gel shift complex seen with this site. This lack of specificity may help explain why AD-HMG I fails to activate the core IgκB reporter in yeast, even though it binds oligonucleotides containing this site \textit{in vitro}. Namely, because HMG I shows only a slight preference in binding to core IgκB NF-κB site-containing oligonucleotides over nonspecific DNA, there is little driving force \textit{in vivo} for AD-HMG I to localize itself on core IgκB site in the presence of a vast excess of nonspecific sites (43). The increased specificity of HMG I for the core PRDII site is apparently sufficient to...
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overcome competition by nonspecific sites in vivo, thus allowing AD-HMG I to activate core PRDII NF-κB site-driven gene expression in yeast.

In an attempt to improve the discrimination of HMG I-His6 for core PRDII versus IgκB sites, we increased the concentrations of nonspecific competitors in the binding reactions by 10-fold (1 mg ml⁻¹ BSA, 50 μg ml⁻¹ poly(d(G-C))). Under these conditions, HMG I shows ~4-fold discrimination on PRDII over IgκB core NF-κB sites (data not shown), more in line with the difference inferred from DNase footprints.

Bearing in mind that intramolecular cooperativity has been shown to influence the strength and specificity of HMG I binding to IFN-γ enhancer (5), we used EMSA to characterize HMG I-His6 DNA binding on PRDII-NRDI and IgκB-NRDI sites, in which the centers of the κB and NRDI elements are located one helical turn away from each other. We found that, even at low concentrations of nonspecific competitors (0.1 mg ml⁻¹ BSA, 5 μg ml⁻¹ poly(d(G-C))), HMG I binds PRDII-NRDI ~5-fold more strongly than IgκB-NRDI (data not shown). Comparison of the K_d values for HMG I alone binding to core versus NRDI-linked κB sites reveals that intramolecular κB site/NRDI cooperativity stimulates the interaction of HMG I with the PRDII site (4-fold, 10 versus 40 nM) significantly more than the IgκB site (1.6-fold, 50 versus 80 nM). These results underscore the importance of flanking AT-rich sequences for high affinity binding and strong discrimination by HMG I alone to different κB DNA motifs.

After this work was largely complete, a report appeared in the literature suggesting that the acidic carboxyl-terminal tail of HMG I might be important for sequence specificity of the protein (5), which raised the possibility that the COOH-terminal His-tagged form of the protein used in the initial in vitro phase of this and other studies might not behave identically to wild type HMG I. Therefore, we carried out a series of EMSA assays on NH₂-terminal His-tagged HMG I protein (His₆-HMG I). As shown in Fig. 3B, when a probe concentration of 5 nM is present in the binding reactions, His₆-HMG I showed relatively strong (~10-fold) discrimination for the PRDII-NRDI probe over the IgκB-NRDI probe. At high concentrations of His₆-HMG I protein (100 nM), significant HMG I binding to the IgκB-NRDI probe was also detected. While the data using both His₆-HMG I and HMG I-His₆ are consistent with a cooperative DNA binding mode wherein a single HMG I molecule contacts two DNA sites simultaneously (sigmoidal binding curves with steep slope), the data also indicate that His₆-HMG I is slightly more proficient at binding and discriminating than HMG I-His₆. We therefore used His₆-HMG I for the remainder of the in vitro EMSA experiments reported here.

To probe further the specificity of HMG I, we carried out oligonucleotide competition experiments under stoichiometric binding conditions (probe concentration >> K_d; data not shown). Even in the presence of high concentrations of nonspecific competitors (1 mg ml⁻¹ BSA, 50 μg ml⁻¹ poly(d(G-C))), the His₆-HMG I:PRDII-NRDI complex was specifically competed by IgκB-NRDI or a 44-bp oligonucleotide containing the core IgκB site, but not by a 20-bp core IgκB oligonucleotide duplex. Nevertheless, competition by IgκB-containing competitors was always significantly weaker than by the corresponding PRDII-containing competitors. Interestingly, even nonspecific competitors lacking any AT-rich sequences can compete HMG I binding to some degree.

To assess the effect of NRDI on cooperation between HMG I and NF-κB in vitro, we incubated various amounts of NF-κB p50:p65 RHR heterodimer with fixed amounts of HMG I and DNA probes. The experimental conditions were chosen so as to maximize the difference in binding of His₆-HMG I to the PRDII-NRDI and IgκB-NRDI probes (Fig. 4A). NF-κB by itself binds much more strongly to the IgκB-NRDI site than to PRDII-NRDI (Fig. 4A, compare lanes 11–15 with 1–5, respectively). However, in the presence of His₆-HMG I, NF-κB bound both sites with almost equal strength, thus indicating that His₆-HMG I stimulates NF-κB binding to PRDII-NRDI to a much greater extent (~50-fold) than to IgκB-NRDI (~2-fold). Importantly, we reproducibly observed a subtle difference in mobility between the NF-κB/DNA gel shift bands in the presence and absence of HMG I, suggesting the formation of a ternary NF-κB-HMG I-DNA complex. These results contrast strikingly with those examining cooperativity on core PRDII and IgκB probes (Fig. 4B). Under the same EMSA conditions as in Fig. 4A, NF-κB by itself exhibits similar affinities for core PRDII and IgκB NF-κB probes (Fig. 4B, lanes 1–5 and 11–15). Further, no dramatic stimulation of NF-κB binding to either core κB probe was observed (Fig. 4B, data not shown). It is unclear whether a ternary NF-κB-HMG I-DNA complex was formed, because any potential mobility differences attributable to the presence of His₆-HMG I are too subtle to provide conclusive evidence (compare lanes 6–10 to lanes 1–5 and lanes 16–20 to lanes 11–15).

Taken together, these results strongly suggest that the AT-rich NRDI site greatly enhances the ability of HMG I to differentiate PRDII and IgκB sites, in terms of both HMG I/DNA recognition and HMG I/NF-κB cooperative DNA binding.

It should be pointed out that the discrimination of NF-κB or
Mammalian cells, and this enabled us to probe further the structural basis for these effects.

Alanine-scanning Mutagenesis of HMG I—Two parallel yeast genetic assays were utilized to explore the mechanism underlying the cooperativity of NF-κB and HMG I on PRDII and IgκB motifs: the primary cooperative DNA binding assay measures the transcriptional activity of the NF-κB RHRs together with HMG I on an IgκB-driven lacZ reporter; the secondary HMG I/DNA binding assay quantifies the activity of HMG I on a lacZ reporter driven by PRDII site. In each case, the choice of NF-κB site was made on the basis of that which was found to yield the most sensitive readout in preliminary experiments. By comparing the effects of mutations in HMG I on these two assays, we can discern those that affect DNA binding of HMG I alone and those that affect cooperativity with NF-κB.

To further determine the influence of the NRDI site on the interaction interface of NF-κB/HMG I on the core PRDII or IgκB NF-κB sites, we compared yeast genetic analyses using core κB reporters with those having a juxtaposed NRDI element.

To map the region within HMG I important for the cooperative NF-κB/HMG I DNA complex formation, we expressed a series of HMG I deletion mutant proteins in yeast. Immunoblotting analysis confirmed that approximately equal amounts of proteins were produced (data not shown). We first tested each mutant protein for its ability to activate the IgκB-NRDI reporter in the presence of NF-κB or to activate the PRDII-NRDI reporter by itself (Fig. 5A). Deletions of the acidic carboxyl-terminal region (residues 91–107) had little effect on either cooperative binding or DNA recognition, but when all the amino acids preceding the first basic repeat (residues 20–31) were deleted, a more than 3-fold loss of activation in the HMG I/PRDII-NRDI recognition assay, in agreement with the reported intramolecular cooperativity of the two repeats in binding to PRDII and NRDI sites (5, 21).

Mutations of several other regions including the first repeat affected both cooperativity and DNA binding to a similar extent. Only 1 block mutant (residues 6–9) showed different behavior in that it activates PRDII-NRDI reporter more strongly than the wild type protein, but does not cooperate with NF-κB as well as wild type HMG I. Consistent with the deletion studies described above, residues 6–9 fall within the region (amino acids 1–19) that has been shown important for cooperation with NF-κB on the IgκB-NRDI reporter. Block alanine mutagenesis studies on core κB reporters yielded slightly different results. The cooperative DNA binding assay with a single IgκB site-driven reporter is less sensitive to mutations in HMG I residues than with a single IgκB-NRDI site-driven reporter: only 3 out of 12 block alanine mutants tested showed more than 3.5-fold loss of transcriptional activity in this assay (Fig. 6B); these are a subset of those that strongly affected cooperation with NF-κB in activation from the core IgκB or IgκB-NRDI reporter (Fig. 6). Alanine substitution was chosen so as to eliminate interactions beyond the side chain ω-carbon, while minimizing the perturbation of secondary structure (32). On the IgκB-NRDI reporter (Fig. 6A), 5 (starred) of the 13 block alanine mutants, together spanning amino acids 1–90, showed greater than 6-fold loss of transcriptional activity in the NF-κB/HMG I/IgκB-NRDI cooperative binding assay; these residues lie within the second and third basic repeats. These same 5 block alanine mutations also dramatically reduced the levels of activation in the HMG I PRDII-NRDI recognition assay, in agreement with the reported intramolecular cooperativity of the two repeats in binding to PRDII and NRDI sites (5, 21).

Mutations of several other regions including the first repeat (residues 21–31) and the threonine-rich region (residues 71–79) affected both cooperativity and DNA binding to a similar extent. Only 1 block mutant (residues 6–9) showed different behavior in that it activates PRDII-NRDI reporter more strongly than the wild type protein, but does not cooperate with NF-κB as well as wild type HMG I. Consistent with the deletion studies described above, residues 6–9 fell within the region (amino acids 1–19) that has been shown important for cooperation with NF-κB on the IgκB-NRDI reporter. Block alanine mutagenesis studies on core κB reporters yielded slightly different results. The cooperative DNA binding assay with a single IgκB site-driven reporter is less sensitive to mutations in HMG I residues than with a single IgκB-NRDI site-driven reporter: only 3 out of 12 block alanine mutants tested showed more than 3.5-fold loss of transcriptional activity in this assay (Fig. 6B); these are a subset of those that strongly affected cooperation with NF-κB in activation from the core IgκB or IgκB-NRDI reporter (Fig. 6). Alanine substitution was chosen so as to eliminate interactions beyond the side chain ω-carbon, while minimizing the perturbation of secondary structure (32). On the IgκB-NRDI reporter (Fig. 6A), 5 (starred) of the 13 block alanine mutants, together spanning amino acids 1–90, showed greater than 6-fold loss of transcriptional activity in the NF-κB/HMG I/IgκB-NRDI cooperative binding assay; these residues lie within the second and third basic repeats. These same 5 block alanine mutations also dramatically reduced the levels of activation in the HMG I PRDII-NRDI recognition assay, in agreement with the reported intramolecular cooperativity of the two repeats in binding to PRDII and NRDI sites (5, 21).
cooperative activation on the NRDI-linked site. It is worth noting that, of the three basic repeats in HMG I, mutations in the first and the third repeats result in only 2-fold reduction of transcriptional activities in both the cooperativity assay and DNA binding assay; mutations in the second repeat had much greater effects (more than 3.5-fold), thus identifying the second repeat as being most important for DNA binding and cooperation with NF-κB on core κB motifs.

To delineate the precise positions within the 3 blocks in which alanine mutations had the most deleterious effects on cooperative binding, we analyzed mutant proteins containing double and single alanine substitutions in this region (residues 53–68; Fig. 6C). On both κB and κB-NRDI reporters, a direct correlation is observed between the transcriptional activities of mutant HMG I proteins in the NF-κB/HMG I/DNA cooperativity assay and in the HMG I/DNA binding assay. This indicates that all the residues within HMG I that are critical for cooperation with NF-κB on core κB site are also important for the HMG I/DNA interaction. No mutations within the second repeat were observed to affect only cooperativity between HMG I and NF-κB, without affecting DNA recognition by HMG I alone; such cooperativity specific mutations have only been identified in the region (residues 1–19) of HMG I. The importance of these residues identified in vivo was partially con-

**FIG. 6.** Transcriptional activities of HMG I polypeptides containing block alanine mutations on (A) κB-NRDI-driven lacZ reporters or (B) core κB-driven reporters. Residue numbers at left in both panels denote blocks that were mutated to alanines. Stars denote mutations that have the strongest effects. C, yeast genetic assays of HMG I polypeptides containing double or single alanine substitutions within the second basic repeat on κB-NRDI or core κB sites. Note that the response of transcriptional activity to alanine mutations is similar in both the cooperative and isolated DNA binding assays. The reporters and β-galactosidase substrates are used in the same way as described in the legend to Fig. 5.
firmed by in vitro quantification of mutant HMG I DNA binding affinities (data not shown).

In an effort to determine whether the mono- and di-alanine mutations affect HMG I/NF-κB interactions in addition to their effects on HMG I/DNA binding, we performed GST fusion protein-protein interaction assays. GST-HMG I fusion proteins (wild type or alanine mutants) bound resins were tested for their abilities to precipitate purified NF-κB p50 RHR (1–366) protein (Fig. 7). Wild type HMG I, as well as 3 block alanine mutants (residues 21–26, 46–52, and 71–74) were found to bind p50 relatively well, although this interaction is abolished with the other alanine mutants (G59A, R60A, P61A4, K62A, and G63A) and GST as a control. The fact that mutations within the second repeat (residues 53–67) can affect the association of HMG I with NF-κB p50 raises the possibility of a dual role for the second repeat in DNA recognition and NF-κB p50 interaction.

**DISCUSSION**

The enhancerome model for transcriptional activation emphasizes the importance of cooperative interactions among regulatory proteins and the transcriptional apparatus as a means of stabilizing an initiation-competent complex (23, 24). HMG I(Y) is unique in several respects among cooperative partners: (i) the protein is exceptionally broad in its DNA sequence preferences; (ii) HMG I(Y) contains no intrinsic transcriptional activation domain, and most probably acts only to enhance the affinity of its partner protein for DNA; and (iii) HMG I(Y) cooperates with an unusually diverse array of structurally unrelated proteins, including NF-κB, ATF-2/c-Jun, Tst-1, and Elf-1 (8–11). A major challenge is to understand how HMG I exerts such specific effects on transcription while exhibiting such apparent promiscuity in binding. The present studies were aimed at gaining insight into these issues through in vitro and in vivo analyses of HMG I and one of its cooperative partners, NF-κB.

An intriguing clue to the mystery of specificity in HMG I/DNA interactions was gained by the observation that the tandem repeats in HMG I can interact simultaneously with appropriately phased AT-rich tracts in the interferon-β promoter (5). This intramolecularly cooperative binding mode was found to enhance the affinity of HMG I/DNA interactions, thereby raising the possibility that specificity might also be affected. Indeed, our results confirm the fact that intramolecular cooperativity substantially strengthens HMG I/DNA interactions; furthermore, we find that the specificity of HMG I for tandem AT-rich sites is significantly increased over that for single sites alone. This dichotomy in DNA-binding mode by HMG I may help to explain the weak discrimination for the core PRDII versus IκB sites observed in our studies, while others have reported stronger discrimination. A critical difference between these studies lies in the fact that we purposely excluded AT-rich flanking sequences from our core κB probes, whereas the naturally occurring DNA elements used previously contained such AT-rich flanking elements as a natural consequence of proper binding function. Remarkably, HMG I shows less than 5-fold discrimination for DNA containing a single AT-rich tract (PRDII) over mixed sequence DNA containing no AT-tract, but increases to nearly 20-fold when the AT-rich tract is linked to another in NRDI.

The effect of tandem intramolecular binding by HMG I on its cooperation with NF-κB has not been examined previously. Here we find that the binding of HMG I to either the core PRDII or IκB site does little if anything to stimulate NF-κB binding (Fig. 4B). By contrast, HMG I powerfully stimulates the binding of NF-κB to the tandemly arrayed PRDII-NRDI site (Fig. 4A). This stimulation does not appear to result simply from tandem intramolecular binding of HMG I, but rather in some more complex way from the DNA sequence, as little cooperativity between HMG I and NF-κB was evident on the IκB-NRDI site (Fig. 4A).

What is the mechanism of cooperative DNA binding by HMG I and NF-κB on PRDII-NRDI? The near perfect correlation between the effects of mutations on reporter assays driven by HMG I alone and in the presence of NF-κB is most consistent with an indirect readout mechanism, wherein the binding of HMG I alters DNA structure so as to make it more favorable for NF-κB recognition. Indeed, the PRDII site contains an intrinsic bend, which is reversed upon NF-κB binding (22); perhaps HMG I lowers the energetic cost of this reversal. Paradoxically, however, several alanine mutations that disturb HMG I-DNA binding and HMG I/NF-κB cooperation also weaken protein-protein interactions between HMG I and NF-κB (Fig. 7), suggesting the two proteins might cooperate through direct contacts as well. This paradox could be resolved if the very same residues in the second basic repeat of HMG I that contact DNA also contact NF-κB. As no experimental data are presently available to address this question, nor was a simple, direct and definitive experimental test immediately apparent, we turned to the NMR structure of the HMG I (51–90) fragment bound to DNA (6). As revealed by this structure, a central Arg-Gly-Arg core (residues 58–60) in the second repeat of HMG I adopts an extended conformation and penetrates deep into the DNA minor groove. Importantly, NF-κB is bound exclusively in the major groove on the backside of DNA. From the perspective of this NMR structure, the spatial separation between the HMG I- and NF-κB-binding sites renders it exceedingly difficult, if not impossible, for multiple DNA contact residues of the HMG I second basic repeat to simultaneously contact residues of NF-κB. Either of two possibilities seems more likely: (i) protein-protein contacts between HMG I and NF-κB that are responsible for GST pull-down result from some weak interaction between the two proteins that bears no relevance to cooperative complex formation; or (ii) perhaps the overall structure of HMG I changes substantially with respect to DNA in going from the binary to ternary complexes, thereby allowing the protein to interact with both DNA and NF-κB using the second basic repeat.

If NF-κB and HMG I cooperate by making mutually favorable alterations of DNA structure, then it is possible to rationalize straightforwardly the marked sequence dependence of cooperativity observed in our in vitro cooperativity assays (Fig. 4A). Specifically, binding of HMG I or NF-κB to one site may induce a DNA structure that favors binding of the other protein, whereas a different DNA sequence cannot adopt the same structure. It is noteworthy that one of the two base pairs that differ in the PRDII and IκB sites is contacted directly by the
p50 subunit of NF-κB: K244 of p50 hydrogen bonds to the T residue of the 5'-GGGAAATTCG-3'. The second repeat (PRDII) or to the corresponding Q residue in IgκB. Likewise, the NMR structure of HMG I peptide bound to DNA reveals that residues Arg-58, Gly-59, and Arg-60 (second repeat) or Arg-84, Gly-85, and Arg-86 (third repeat) make extensive minor groove contacts to both base pairs that differ in PRDIV and IgκB (6).

The fact that both partners contact the same base pairs in PRDIV and IgκB, although from different grooves, provides a clear mechanism for the two partners to communicate with one another through DNA. It stands to reason that the ability to communicate and thus bind DNA cooperatively should depend upon the covalent structure and conformation of the bound DNA sequence.

The inability to pinpoint precisely the mechanism of cooperativity between HMG I and NF-κB reflects in no small part the inherent difficulty of studying proteins such as HMG I that exhibit relatively weak specificity, and possess little or no secondary or tertiary structure, even when bound to DNA. There exists a great need for new experimental approaches to study such problems. It is worth mentioning that even alanine-scan mutagenesis fails to detect any potentially important interactions modes of the second and the third repeats with DNA that have been illustrated in the HMG I (51–90)/DNA NMR structure (6).

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