The mammalian transcription factor, NF-Y(CBF), contains three known subunit components, NF-YA (CBF-B), NF-YB(CBF-A), and NF-YC(CBF-C), which are all required to reconstitute specific CCAAT box DNA binding activity. In this study, the high mobility group chromosomal protein, HMG-I(Y), has been shown to activate NF-Y in transient transfections in vitro using the natural murine α2(I) collagen promoter and a multimerized version of the proximal NF-Y(CBF) CCAAT box element. In vitro analysis of the α2(I) collagen promoter region inclusive of the NF-Y(CBF) binding site (−106 to −65 base pairs) failed to identify any high affinity HMG-I(Y) DNA-binding sites. However, the heterotrimeric NF-Y complex, as well as the NF-YA subunit alone, was shown to stably interact in vitro with both HMG-I(Y) and phosphorylated HMG-I, as modified by casein kinase II, using far Western and protein-protein interaction solution assays in the absence of CCAAT box DNA. Furthermore, the interaction between HMG-I(Y) and NF-Y was mapped to the highly conserved DNA binding-subunit interaction domain (DBD) of the NF-YA subunit and to a single AT-hook motif in HMG-I(Y). Recombinant HMG-I was also found to stabilize the CCAAT box DNA binding activity of recombinant NF-Y, as well as the native NF-Y complex, in vitro. Together, these results suggest a functional HMG-I(Y) protein binding site has been identified in the NF-Y complex and mapped to the conserved DBD and AT-hook regions of NF-YA and HMG-I(Y), respectively. This protein-protein interaction site may function to modulate NF-Y activity through stabilization of NF-Y binding to its CCAAT box DNA-binding site.

Nuclear factor-Y (NF-Y)1, also referred to as the CCAAT-binding factor, CBF (2), utilizes the highly conserved regions located in three heterologous subunits to create a DNA binding-subunit interaction domain (DBD) that specifically recognizes a CCAAT box motif found in the promoter and enhancer regions of many eukaryotic genes (3). The Y box element of all murine major histocompatibility complex (MHC) class II promoters, for example, contains a highly conserved inverted CCAAT DNA sequence whose conservation extends into the flanking regions, as well as its overall physical location in relation to the highly conserved 5′ X box element, and plays a critical role in MHC class II gene transcription (4). The human and murine NF-YABC DBD elements have been strongly conserved in evolution (5), are highly related to the corresponding regions in the Saccharomyces cerevisiae HAP2/3/5 CCAAT box factor complex, and can be functionally interchanged with these yeast subunits (6, 7). Recently, the DBD regions of the NF-YB and NF-YC subunits have been suggested to interact through a protein-protein histone-fold “handshake” motif (8, 9) in a manner analogous to the histone proteins, H2B and H2A, respectively (10). In addition, the NF-YA(CBF-B) subunit has been shown to interact with the NF-YB(CBF-A):NF-YC(CBF-C) heterodimer and neither of these subunits alone suggesting that a unique interaction structure for the NF-YA(CBF-B) subunit is created through conformational changes associated with dimerization of NF-YB(CBF-A):NF-YC(CBF-C) (7).

HMG-I(Y) belongs to a group of abundant low molecular mass non-histone chromosomal proteins that possess three copies of a reiterated 9-amino acid motif (the A′T hook) that interacts with the minor groove of many AT-rich DNA sequences (11). These proteins are soluble in 5% perchloric acid, resistant to heat denaturation, can alter DNA structure, and have been shown to be important regulators of gene transcription as well as involved in chromatin structure. HMG-I(Y) proteins are substrates for p34
\(^{\text{cdk2}}\)/cyclin B kinase (12) and casein kinase II (CKII) (13, 14), and in both cases phosphorylation significantly decreases specific DNA binding activity. HMG-I(Y) DNA-binding sites have been identified in functional regions of many gene promoters which include interleukin-4 (15), interleukin-2 receptor α-chain (16), lymphotixin (17), the human papovavirus JC (18), HLA DRα (19), and the CD28 response elements within granulocyte-macrophage colony-stimulating factor and interleukin-2 (20). These sites are often found in close proximity to the DNA-binding sites of known transcription factors (e.g. NF-κB (21), Tst-1/Oct-6 (18)), and in particular are critical for viral induction of the human IFN-β gene (22–24). In addition, HMG-I(Y) has been shown to bind to the basic leucine zipper region of activating transcription factor-2 (ATF-2) (19) which promotes the dimerization of this factor and stimulates its binding to the IFN-β promoter (25). These observations suggest that HMG-I(Y) plays a critical role in IFN-β gene regulation through a combination of effects on transcription factor subunit interactions, factor binding stabilization, DNA bending, exclusion of specific factor DNA binding (e.g. ATF-2) (26), and assembly of a multi-component enhanceosome (24). HMG-I(Y) binding to the highly conserved regions of Elf-1 (Ets domain)
(16) and Tst-1/Oct-6 (POU domain) (18) through protein-protein interactions have been identified. These studies suggest that these DNA-independent HMG-I(Y) protein binding sites also play important overall functional roles in modulating specific transcription factor transactivation potential.

Type I collagen is composed of a heterotrimer of two \( \alpha 1 \) and one \( \alpha 2 \) subunits and is the predominant fibrillar collagen protein present in bones and tendons. The transcriptional regulatory elements of the murine \( \alpha 2(1) \) collagen promoter have been extensively investigated using both transient transfection, transgenic animal, and \emph{in vitro} cell-free transcription approaches, and the proximal CCAAT box motif at \(-87\) nucleotides has been shown to play an important functional role in its cellular regulation (26–28). In this report, the murine \( \alpha 2(1) \) collagen promoter and multimerized version of the \( \alpha 2(1) \) collagen NF-Y(CBF) binding site have been used to investigate the role of HMG-I(Y) as a protein cofactor in modulating NF-Y function. HMG-I has been shown to activate NF-Y \emph{in vivo} and to interact with the highly conserved DBD region of NF-Y \emph{in vitro}. These results represent the first report of an additional functional protein component associated with the NF-Y complex besides its core YA/B/C subunits and suggest that HMG-I(Y) may modulate NF-Y activity through direct association with the DBD in the NF-YA subunit.

**MATERIALS AND METHODS**

**Recombinant Plasmids**—The \( \alpha 2(1) \) collagen promoter, pH6 (26), was used as template to generate a NF-Y(CBF) CCAAT box site-directed mutation with the Quick-Change mutagenesis technique as described by the manufacturer (Stratagene, La Jolla, CA). Both the wild-type and NF-Y mutant HindIII fragments, which contain the \( \alpha 2(1) \) collagen promoter derived from pH6, were isolated and cloned into the HindIII site of the pGEX2 vector (Promega) to generate pH6 GL3 and pH6m GL3, respectively.

Cloning of full-length human and murine NF-YA, YB, and YC subunits into the pGEX2T vector (Pharmacia Biotech Inc.) has been described previously (5). BanHI-EcoRI fragments of each subunit were cloned into the pGEX2TK vector to generate pH6m GL3 and pH6-GL3, respectively.

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The following plasmids were kindly provided as follows: pH6, pFC1, pH6m GL3, and pFC2 (26, 27).

**Materials and Methods**

**Recombinant Plasmids**—The \( \alpha 2(1) \) collagen promoter, pH6 (26), was used as template to generate a NF-Y(CBF) CCAAT box site-directed mutation with the Quick-Change mutagenesis technique as described by the manufacturer (Stratagene, La Jolla, CA). Both the wild-type and NF-Y mutant HindIII fragments, which contain the \( \alpha 2(1) \) collagen promoter derived from pH6, were isolated and cloned into the HindIII site of the pGEX2 vector (Promega) to generate pH6 GL3 and pH6m GL3, respectively. BanHI-EcoRI fragments of each subunit were cloned into the pGEX2TK vector to generate pH6 GL3 and pH6m GL3, respectively. pGEX2TK vector (Pharmacia Biotech Inc.) has been described previously (5). Anti-HMG-I polyclonal antibodies were kindly provided as described previously (30). Anti-HMG-I polyclonal antibodies were prepared as described previously (36). The following oligonucleotides were used to generate the NF-Y(CBF) CCAAT box mutation in the \( \alpha 2(1) \) collagen promoter of pH6: 5'-TACCGTTAGCTTAAGGCTTGAGGGC-3' and 5'-GGGCCATCCACATAGGGCTGAGGGC-3', where the introduced XhoI site is underlined. The "small" murine \( \alpha 2(1) \) collagen proximal promoter NF-Y CCAAT probe (S-collagen) was prepared with 5'-GGCCCT CGCCCTCCCTCACCTTTGAGGGC-3' and 5'-GGGCGTCTCCACCATCGGGCGG-3', where the "large" murine \( \alpha 2(1) \) collagen proximal promoter NF-Y CCAAT probe (L-collagen) was prepared with 5'-CCATCGCCCTCACCACTGAGAGG-3' and 5'-GGGCGTCTCCACCATCGGGCGG-3'. The NF-Y(CBF) CCAAT box binding site motif is underlined above the S- and L-collagen and Ec0 DNA probes. The NF-Y(CBF) CCAAT box binding site probe was derived from the human interferon-\( \beta \) (IFN-\( \beta \)) promoter, PRDI (23) with 5'-GCCCTGACCTTACCTGAGGG-3' and 5'-GGGCGTCTCCACCATCGGGCGG-3'. The "large" murine \( \alpha 2(1) \) collagen proximal promoter NF-Y CCAAT probe (L-collagen) was prepared with 5'-CCATCGCCCTCACCACTGAGAGG-3' and 5'-GGGCGTCTCCACCATCGGGCGG-3'. The "small" murine \( \alpha 2(1) \) collagen proximal promoter NF-Y CCAAT probe (S-collagen) was prepared with 5'-GCCCTGACCTTACCTGAGGG-3' and 5'-GGGCGTCTCCACCATCGGGCGG-3'. The NF-Y(CBF) CCAAT box binding site motif is underlined above the S- and L-collagen and Ec0 DNA probes. The NF-Y(CBF) CCAAT box binding site probe was derived from the human interferon-\( \beta \) (IFN-\( \beta \)) promoter, PRDI (23) with 5'-GCCCTGACCTTACCTGAGGG-3' and 5'-GGGCGTCTCCACCATCGGGCGG-3'. The "large" murine \( \alpha 2(1) \) collagen proximal promoter NF-Y CCAAT probe (L-collagen) was prepared with 5'-CCATCGCCCTCACCACTGAGAGG-3' and 5'-GGGCGTCTCCACCATCGGGCGG-3'. The "large" murine \( \alpha 2(1) \) collagen proximal promoter NF-Y CCAAT probe (L-collagen) was prepared with 5'-CCATCGCCCTCACCACTGAGAGG-3' and 5'-GGGCGTCTCCACCATCGGGCGG-3'. The "large" murine \( \alpha 2(1) \) collagen proximal promoter NF-Y CCAAT probe (L-collagen) was prepared with 5'-CCATCGCCCTCACCACTGAGAGG-3' and 5'-GGGCGTCTCCACCATCGGGCGG-3'. The "large" murine \( \alpha 2(1) \) collagen proximal promoter NF-Y CCAAT probe (L-collagen) was prepared with 5'-CCATCGCCCTCACCACTGAGAGG-3' and 5'-GGGCGTCTCCACCATCGGGCGG-3'. The "large" murine \( \alpha 2(1) \) collagen proximal promoter NF-Y CCAAT probe (L-collagen) was prepared with 5'-CCATCGCCCTCACCACTGAGAGG-3' and 5'-GGGCGTCTCCACCATCGGGCGG-3'...
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column was developed using a step KCl gradient, and NF-Y was eluted in the BC-420 fraction (0.4 M KCl). This fraction was further dialyzed against BC-100 and stored in aliquots at −80 °C. Depleted HeLa nuclear extracts retain NF-Y but lack HMGI-Y binding activity as measured by EMSA using the IFN-β probe. To prepare the HeLa YAF fraction, insoluble nuclear extracts were prepared as described above, and then the flow-through fractions were centrifuged through Centricron 30 filtration devices (Amicon, Beverly, MA). The filtrate fraction was diluted to 0.1 M KCl and the buffer exchanged to desalted form as described previously, and then the flow-through fractions were centrifuged at 100,000 rpm for 10 min at 4 °C. The pH of the supernatant was adjusted to 7.5 with 10 N NaOH and then dialyzed against 1 M non-fat dry milk, 0.1% Nonidet P-40). Filters were washed 5 times with 6, 3, 1.5, and 0 M guanidine HCl solutions that contained 0.5% Nonidet P-40 at 4 °C. SDS-PAGE sample buffer (100 μl) was added, and the samples were heated to 95 °C. Aliquots of each bead reaction (20 μl) were separated on SDS-PAGE gels. Dried gels were exposed to XAR-5 film at room temperature. In experiments involving interaction of affinity purified NF-Y and GST fusion proteins, glutathione-agarose beads were washed five times with 1 ml of BC-100 each following the 1-h binding reaction at 4 °C. Proteins were eluted using BC-420, concentrated, and the buffer exchanged to BC-100 using Centricon 10 filtration devices. Eluted fractions were analyzed using EMSA.

RESULTS

The High Mobility Group Protein HMG-I Activates NF-Y in Vitro—Biochemical characterization of the native NF-Y transcription factor complex in a variety of human and murine cell lines that represent distinct stages of B lymphocyte development and adipocyte differentiation has suggested that the NF-YABC complex can associate with a group of newly identified protein cofactors (YAF's, NF-Y Associated Factors). The partially purified YAF fraction derived from mature B cells, for example, has been shown to confer the biochemical property of high affinity anion exchange binding to NF-Y derived from plasma B cells and several other MHC class II cell lines (e.g., HeLa, 3T3-L1). Further biochemical analyses have suggested the YAF fraction derived from a variety of cell types (e.g., HeLa) contains an HMG-I(Y)-like activity as defined by specific interaction with a known IFN-β HMGI(Y)-DNA binding site, solubility in 5% perchloric acid, resistance to denaturation and loss of DNA binding activity following exposure to elevated temperature (e.g., 65 °C), and competition of IFN-β DNA binding activity by poly(dl-dC) DNA and specific α-HMG-I antibodies (Fig. 1). These observations have led to consideration of the possibility that HMG-I(Y) is in fact one of the YAF proteins and is involved in regulating NF-Y function through direct protein-protein interactions.

The well-characterized murine a2(I) collagen promoter (2, 26–28) was chosen to assess the functional effect of HMG-I on NF-Y-mediated transcription in vivo. A multimerized NF-Y(CBF) reporter construct derived from the murine a2(I) collagen promoter, pFC1, was also tested since a number of studies have demonstrated the relatively low in vitro activity of single and multimerized NF-Y binding site reporters derived from MHC class II promoter Y box elements in lymphocyte cell lines (21, 42), and pFC1 has been shown to exhibit moderate relative reporter activity in a variety of cell types (26–28). As shown in Fig. 2 expression of HMG-I in HeLa cells resulted in an ~6-fold activation of the natural a2(I) collagen promoter, and an ~2.7-fold activation of the multimerized NF-Y site reporter, pFC1. In contrast, this level of HMG-I expression did not activate the NF-Y purifying mutant reporter, pFC GL3, or the mutant multimerized NF-Y site reporter, pFC2. These results suggest that HMG-I modulates the functional activation of the natural a2(I) collagen promoter directly through NF-Y and its binding to this CCAAT box motif since disruption of the NF-Y(CBF) binding site in both pHe6m GL3 and pFC2 resulted in no activation by CMV-HMG-I.

In an attempt to identify potential HMG-I(Y) DNA-binding sites in the murine a2(I) collagen promoter comparative EMSA

2 R. A. Currie, unpublished data.
analyses were performed using regions contained in pFC1 (Fig. 3). The α2(I) collagen DNA probes, L-collagen (−101 bp to −65 bp) and the S-collagen probe (−98 to −72 bp), were both shown to specifically bind HeLa cell NF-Y (panels A and B respectively, lanes 1–4) as compared with a control MHC class II Eα NF-Y binding site (lanes 5–8). L- and S-collagen probes were also compared with a well characterized HMG-I(Y) DNA-binding site from the IFN-β PRDII element (23) and to the AT-rich MHC Eα probe (36). Recombinant GST-HMG-I was observed to bind to the L-collagen probe with very low affinity (panel A, lane 9) in comparison to the IFN-β (lane 11) and Eα (lane 13) probes, and GST-HMG-I was not observed to bind to the S-collagen probe (panel B, lane 9), whereas high affinity binding of GST-HMG-I to the control IFN-β probe was observed (lane 13). To extend these observations further highly purified recombinant HMG-I expressed from the pET15b vector was also tested, and HMG-I binding to either the L-collagen (panel C, lane 2) or S-collagen (panel C, lane 5) probes was not observed. Together these results suggest that the α2(I) collagen promoter region in pFC1 (−101 to −65 bp) which contains the functional NF-Y(CBF) site does not contain high affinity HMG-I(Y) DNA-binding sites.

Physical Interaction between NF-Y and HMG-I(Y)—The previous analyses suggested that HMG-I(Y) may function to activate NF-Y through direct protein-protein interactions. This hypothesis was examined using a far Western blot analysis in Figs. 4 and 5. The full-length NF-YA subunit was first 32P-labeled, recombined with excess purified NF-YB and NF-YC subunits in vitro, and the 32P-NF-Y complex was used to probe a nitrocellulose filter which contained GST, HMG-I and several other GST-fusion transcription factors (Fig. 4, panel A). Strong interaction of the 32P-NF-Y probe with both HMG-I(lane 2) and the general transcription cofactor, PC4/p15 (31, 43) (lane 5), was observed. No interaction was observed with GST (lane 1) or several other well characterized transcription factors (lanes 3, 4, and 6). Full-length 32P-NF-YB was recombined with excess purified NF-YA and NF-YC subunits in a similar manner and tested as described above; again, a strong interaction was observed between this 32P-NF-Y complex and both HMG-I and PC4/p15 (panel B). In an attempt to determine if all three NF-Y subunits are required for these in vitro interactions, individual subunits were labeled and tested, alone or in specific NF-Y subunit combinations in far Western analyses. Full-length 32P-YA was observed to specifically interact with both HMG-I and PC4/p15 (panel C), whereas the individually labeled subunits (32P-YB, 32P-YB(DBD), 32P-YC, 32P-YC(DBD)) or the heterodimeric complexes (32P-YB-YC complex and 32P-YB (DBD)-YC(DBD)) interacted in a strong nonspecific manner with the control GST protein, all GST fusion transcription factors, and low abundant bacterial proteins present in these preparations. Both NF-YB and NF-YC subunits contain several hydrophobic regions (7), are tightly associated with each other (44), and may form many nonspecific protein interactions when analyzed in this manner using far Western assays. Together these results suggest that the intact NF-Y complex is capable of stable interaction with HMG-I and PC4; however, the NF-YA subunit directly mediates these interactions, and its contact with HMG-I and PC4 does not depend on additional interactions with NF-YB and NF-YC. As an additional control for these experiments, 32P-GST was tested against the panel of proteins described in panel A and failed to interact with HMG-I, PC4, or any of the other GST fusion proteins.

The ability of full-length NF-YA to interact with HMG-I in vitro suggested that the highly conserved DNA binding-subunit interaction domain (DBD) of YA (5, 7) could contain the region which stably bound both HMG-I and PC4. To test this possibility, the YA(DBD) deletion mutant was first used to probe a panel of GST fusion proteins (panel D). The N-terminal activation domain of NF-YA, YA(DBD), did not form a stable complex with HMG-I (lane 2), PC4 (lane 3), or any other transcription factor tested (lanes 4–6) and suggested that the DBD element of NF-YA was sufficient for the physical interaction between HMG-I and PC4 as measured by far Western analysis.

Mapping of Regions in NF-Y and HMG-I(Y) That Are Necessary for Protein-Protein Interaction—HMG-Y is identical to HMG-I in composition except HMG-Y has an 11-amino acid region deleted as a result of an alternative mRNA splicing event (11, 23). A previous study has identified a small 10-amino acid region in HMG-Y that is responsible for physical interaction with the Tst-1/Oct-6 POU domain (18). To more accurately map the region in HMG-I(Y) that interacts with NF-Y wild-type HMG-Y, this series of HMG-Y mutants was analyzed.
using far Western analysis (Fig. 5). Both full-length NF-YA in the complete NF-Y complex (panel A) and the full-length NF-YA subunit alone (panel B) stably interacted with HMG-I(Y) and PC4. HMG-I(Y) mutants H20–56 and H32–46 which essentially contain AT-hook motifs 1 and 2 and AT-hook motif 2, respectively, formed stable complexes with these 32P-NF-Y and 32P-YA probes. However, neither of these probes recognized the HMG-I(Y) mutant (H46–56) that interacts with the Tst-1/Oct-6 POU domain and contains only a 10-amino acid portion of AT-hook motif 2. These results suggest that at least one intact HMG-I(Y) AT-hook motif is required for stable interaction with either the NF-Y complex or the NF-YA subunit and more specifically suggest that amino acids 32–46 of HMG-I(Y) are sufficient for interaction with NF-Y. In addition, these probes failed to recognize the PC4 mutant (ΔC103–127) that lacks its C-terminal 35 amino acids (panels A and B, lane 7, respectively). To provide further evidence that the DBD element of NF-YA interacts with HMG-I(Y), the NF-YA(DBD) subunit was 32P-labeled and tested together with YB(DBD)/YC(DBD) in the NF-Y(DBD) complex (panel C) and also independently (panel D). Both of these probes recognized full-length HMG-I(Y); however, interaction with all other HMG-I(Y) mutants was severely impaired. These results suggest that the YA(DBD) element is sufficient for stable interaction with the full-length HMG-I(Y) and that an intact AT-hook is sufficient for stable interaction with the NF-Y complex composed of its full-length subunits.
HMG-I Stabilizes NF-Y Interaction with CCAAT Box DNA Elements—Previous studies in a variety of systems have demonstrated that HMG-I(Y) is capable of stimulating the DNA binding activity of NF-κB, Tst-1/Oct-6, ATF-2, and Oct-2A generally under conditions of dilute protein concentration (18, 19, 23, 25). In the case of ATF-2, HMG-I has been shown to also stimulate ATF subunit dimerization (25). To determine if HMG-I is capable of stimulating NF-Y binding by a similar mechanism, the effect of recombinant HMG-I on NF-Y DNA binding activity was analyzed (Fig. 6). Under conditions of low relative NF-Y subunit concentration, HMG-I was observed to specifically stimulate the DNA binding activity of both the His-fusion (panel A) and GST fusion (panels B and C) NF-Y complexes using either the L- or S-collagen probes. The effect of HMG-I on native NF-Y derived from HMG-I(Y) depleted HeLa nuclear extracts was also tested (panel D). In all cases HMG-I was found to dramatically stimulate NF-Y CCAAT box DNA binding activity with no apparent effect on the relative mobility of the DNA-protein complex.

To determine if the native NF-Y complex is capable of interacting with recombinant HMG-I, a glutathione-agarose pull-down assay was performed (Fig. 7). NF-Y was derived from HeLa nuclear extracts, affinity purified, and its CCAAT box DNA binding activity was observed to be stimulated specifically by His-HMG-I (lanes 12–18). NF-Y was incubated with glutathione-agarose beads alone and with GST, GST-HMG-I, and GST-Dr1 bound to beads. NF-Y binding to GST-HMG-I was observed (lane 4), and its CCAAT box activity was likewise stimulated by exogenous His-HMG-I (lane 8). These results suggest that the native NF-Y complex can stably interact with HMG-I in solution and further suggest that HMG-I may represent one of several nuclear factors that contribute to the apparent overall high level of NF-Y CCAAT box DNA binding activity which is observed in unfractionated nuclear extracts.

Phosphorylated HMG-I Interacts with NF-YA in Vitro—Human HMG-I has been shown to be phosphorylated by CKII in its C-terminal region at serine residues 102 and 103 (11), and the DNA binding activity of CKII-treated HMG-I to a known HMG-I(Y) site in the murine Ge promoter has been shown to be...
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The native NF-Y complex interacts with recombinant HMG-I in solution. NF-Y derived from depleted HeLa cell nuclear extracts was affinity purified and incubated with glutathione-agarose beads alone or beads bound with GST, GST-HMG-I, or GST-Dr1 and then eluted and asayed for NF-Y DNA binding activity to the S-collagen probe using EMSA. An aliquot of each eluted fraction was also retained and then incubated with GST and a variety of GST fusion proteins bound to glutathione-agarose beads. The retained 32P-HMG-I fraction was assayed using SDS-PAGE. Lanes 1 and 8, glutathione-agarose beads alone; lanes 2 and 9, GST; lane 3, GST-YAB (His)/YC(His); lanes 4 and 10, GST-YA; lane 5, YB (His)/YC(His); lane 6, GST-DFB; lane 7, GST-Dr1; lane 11, GST-YA(ΔDBD); lane 12, GST-YA(ΔDBD)/GST-YA(ΔDBD); lane 13, GST-HAP2(ΔDBD). Molecular mass markers (New England Biolabs) are denoted at the left in kilodaltons.

Reduced 5-fold (14). In addition, p34cdc2/cyclin B kinase phosphorylates human HMG-I at threonine residues 53 and 78 and has been shown to reduce its DNA binding activity ~20-fold under physiological salt conditions (12). To determine if phosphorylated HMG-I stably interacts with NF-Y in solution recombinant HMG-I was 32P-labeled using CKII and tested using the NF-Y subunits and several additional fusion proteins in a glutathione bead pull-down assay (Fig. 8). 32P-HMG-I (CKII) was found to interact with the assembled NF-YABC complex (lane 3), as well as full-length GST-YA (lanes 4 and 10), GST-YA(ΔDBD) (lane 12), and the highly conserved region of the YB/YC heterodimer (YB/YC(ΔDBD)) (lane 11). Strong interaction between 32P-HMG-I and the general transcription initiation factor, TFIIIB, was also observed (lane 6); however, the functional relevance of this interaction remains unknown at present. p34cdc2/cyclin B-phosphorylated HMG-I was observed to interact in an identical manner to CKII-treated HMG-I in glutathione bead pull-down assays. These results further support the conclusion that HMG-I(Y) interacts with the NF-Y complex through a protein-protein binding site located in the 69 amino acid YA/DBD) region and that phosphorylation of HMG-I by CKII or p34cdc2/cyclin B does not block this interaction.

To support further the conclusion that NF-YA and HMG-I physically interact and that phosphorylation of HMG-I does not promote this interaction, YA(ΔDBD) was cleaved from GST-YA(ΔDBD), 32P-labeled using HMK, and used to probe a set of GST-HMG-Y(I) mutants (Fig. 9). 32P-YA(ΔDBD) was observed to interact with full-length HMG-Y, mutants which contain an intact AT-hook motif, and the single N-terminal AT-hook derived from human HMG-I (lane 13), whereas no interaction was observed with the HMG-Y-(46–56) mutant that contains a disrupted AT-hook motif. In conjunction with far Western analyses, these results suggest that a single AT-hook motif in either HMG-I or HMG-Y is necessary and sufficient to support stable interaction with NF-Y.

DISCUSSION

Ion exchange analysis of the native NF-Y complex in specific states of cellular differentiation has suggested that NF-Y is associated with a group of previously unknown protein cofactors (YaFa). Further characterization of this operationally defined cellular YAF fraction suggested that an HMG-Y-like activity was present and possibly associated with native NF-Y in the absence of CCAAT box binding sites (Fig. 1). Based on these initial biochemical observations, the functional effect of...
Functional Interaction between NF-Y and HMG-I(Y)

Human HMG-I on a well characterized NF-Y(CBF) reporter plasmid derived from the murine α2(I) collagen promoter was tested in vivo (Fig. 2). The murine α2(I) collagen gene has been shown to contain a number of functional DNA elements with associated transcription factors that are involved in regulating its expression in a tissue-specific manner. In particular, the proximal CCAAT box DNA element (−84 bp to −300 bp) has been shown to bind NF-Y(CBF) and to function in a multimerized configuration together with its TATA box and initiation region (−41 bp to +54 bp) in a number of cell types (2, 27, 28). These NF-Y-containing promoter constructs were chosen to examine the possible direct functional effects of HMG-I(Y) on NF-Y both in the context of other transcription factors and in the absence of additional transcription factors as well as the effect of other potential AT-rich HMG-I(Y) DNA-binding sites. Cotransfection of HMG-1 into HeLa cells resulted in activation of the collagen reporters, pH6 GL3 and pFC1, which were dependent on an intact wild-type CCAAT box DNA sequence (Fig. 2). These results suggested that HMG-I(Y) could play a functional role in modulating the transactivation potential of NF-Y in vivo through direct interaction with NF-Y. To assess the possibility that HMG-I was interacting with AT-rich DNA sequences in the α2(I) collagen promoter, a series of EMSA experiments were performed. Using highly purified recombinant HMG-I high affinity HMG-I(Y) DNA-binding sites were not detected (Fig. 5). These observations suggested that activation of the NF-Y reporter by HMG-I could be mediated through direct protein-protein interactions.

To test this possibility far Western assays were performed using both individually labeled NF-Y subunits and NF-YABC complexes that contained one 32P-labeled subunit. The NF-Y complex was consistently observed to specifically interact with the two general transcriptional coactivators, HMG-I(Y) and PC4/p15 (Fig. 4). In addition, the NF-YA subunit alone was capable of stable interaction with HMG-I and PC4, whereas a truncated version of YA that contained its N-terminal activation domains (26) but lacked the highly conserved DDB element was incapable of stable interaction with these two coactivators (Fig. 4, panel D). The interaction of NF-Y with either HMG-I(Y) or PC4 did not depend on additional interactions with the NF-YB or NF-YC subunits. Together these results suggested that the YA DBD element, itself, was necessary and sufficient for these observed protein-protein interactions. Of particular note was the observation that NF-YA and the NF-Y complex each stably interacted with full-length PC4, while deletion of the C-terminal 35 amino acids from PC4 completely disrupted this interaction. PC4 has been shown to be a general accessory factor that is involved in the response of RNA polymerase II to transcriptional activators, both in vivo and in vitro reconstituted systems (31, 43). In addition, phosphorylated PC4 has been shown to be functionally inactive in reconstituted cell-free in vitro transcription assays, whereas the purified native non-phosphorylated and E. coli-derived form of PC4 are potent activators in vitro (43, 45). Mapping of the PC4-NF-Y interaction site to a region in PC4 that is not hyperphosphorylated in vivo (i.e. the extreme N terminus) suggests that PC4 may interact with NF-Y both in its phosphorylated and nonphosphorylated states, and thereby serve to function as both activator and repressor of NF-Y-mediated RNA polymerase II transcription. Further analyses will be directed at uncovering the functional role of PC4 in modulating NF-Y activity in vivo, determining if these phosphorylated forms of PC4 exhibit differential functional effects on NF-Y in specific promoter contexts, and if the coactivators, HMG-I(Y) and PC4, influence each other’s interaction with the YA DBD.

To begin to map the functionally relevant regions in both the NF-Y complex and in the HMG-I(Y) molecule responsible for these interactions, far Western assays were performed using truncated versions of NF-Y subunits and a series of HMG-Y deletion mutants (18) (Fig. 5). An HMG-Y molecule that contained 1 or 2 copies of its AT-hook motif (11) interacted stably with either the full-length NF-YA subunit alone or in the NF-Y complex; however, partial deletion of a single HMG-Y AT-hook motif 2 completely disrupted these interactions. These results suggest that at least one intact copy of the reiterated AT-hook motif is necessary and sufficient for stable interaction with either the YA subunit or the NF-Y complex. To further map the interaction domain in YA the YA(DBD) probe was tested alone and in the NF-Y complex. Stable interaction with full-length HMG-I(Y) was observed in both cases; however, this interaction was severely weakened in mutants that contained 1 or 2 copies but not 3 copies of the AT-hook motif when the YA(DBD) probe was tested alone. In contrast, the HMG-Y mutant with 2 intact AT-hook motifs interacted stably with the intact NF-Y(Y)DBD) complex. The conformation of YA(DBD) in a complete NF-Y complex may be slightly altered in comparison to YA(DBD) alone, and its interaction with these HMG-Y mutants may be facilitated to a greater extent. Analysis of the HMG-I(Y) mutants using the YA (DBD) as probe further supported the conclusion that a single intact AT-hook motif was required for stable interaction (Fig. 9) and suggested that the reiterated AT-hook motif in HMG-I(Y) may function both in DNA bending and in specific protein-protein interactions. NF-YA was not observed to discriminate between particular AT-hook motifs, as human AT-hook (motif 1) and murine AT-hook (motif 2) each were sufficient to support stable interaction. In particular gene contexts a single HMG-I(Y) molecule may function to stabilize transcription factor-DNA binding through a combination of its own binding to a proximal minor groove AT-rich sequence and through direct interaction with an adjacent transcription factor through one of its additional AT-hook motifs or through direct effects on the conformation of multi-subunit proteins, such as NF-Y. These results suggest that HMG-I(Y) interacts with the highly conserved DBD element in the NF-YA subunit, and this interaction possibly maps to a face of the YA α-helix which is not involved in the primary subunit interactions with the
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YB/YC heterodimer (29), since inclusion of the YB/YC subunits in interaction assays neither prevented nor were required for the observed binding. Further mutational analyses will be directed at determining the precise amino acids in the 69-amino acid YA(DBD) element which are critical for HMG-I(Y) interaction and their relationship to the yeast homolog, HAP2(DBD).

In an attempt to identify the biochemical mechanisms that underlie the observed functional effect of HMG-I on NF-Y in vitro, we performed experiments to examine NF-Y DNA binding activity in the presence of HMG-I. Under conditions of low NF-Y subunit concentration, recombinant HMG-I was observed to stimulate NF-Y DNA binding activity to the α2(I) collagen CCAAT box. This effect was specific to HMG-I and also was observed using an HMG-I-depleted HeLa cell nuclear fraction that contained native NF-Y. Co-immunoprecipitation experiments using anti-NF-YB antibodies did not conclusively identify in vivo association of NF-Y and HMG-I.2 In the case of Tst-1/Oct-6 (18), HMG-I has not been shown to alter its EMMA mobility position, whereas slight changes in mobility with NF-xB (23) and ATF-2 (22) have been observed. In these examples HMG-I(Y) has been suggested to stabilize transcription factor-DNA interactions. HMG-I has not been shown to be present in a ternary complex in association with NF-Y or any of the transcription factors identified in these systems in vitro or to be associated with these factors in vivo. HMG-I(Y) may promote transcription factor conformational changes that in turn stabilize overall interaction with their respective DNA-binding sites and only be transiently associated with NF-Y or any of the transcription factors identified in these systems in vitro or to be associated with these factors in vivo. In a related system the Tax protein of human T cell leukemia virus type-1 has been shown to stabilize ATF-DNA binding interactions; however, stable complex interaction was not observed using a variety of EMSA techniques but only using a DNA-co-immunoprecipitation assay (38). Together these studies suggest that ternary complexes in general are sensitive to analysis under EMSA conditions in vitro but in particular instances ternary complexes can be observed in solution. In conclusion, this study provides the first report of an additional protein cofactor functionally associated with the NF-Y complex, maps the interaction site between HMG-I(Y) and NF-Y to a single AT-hook motif and the highly conserved DBD element of the NF-YA subunit, respectively, and suggests that HMG-I can stabilize NF-Y-CCAAT box interactions through direct protein-protein interaction.

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