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DNA binding of NF-Y: the effect of HMGI proteins depends upon the CCAAT box

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Abstract NF-Y is a conserved sequence-specific transcription factor binding to CCAAT boxes. The chromatin-associated HMGI proteins influence promoter activities through positive and negative effects on binding of transcription factors. It was previously shown that HMGI(Y) synergizes the binding of NF-Y to the α2-collagen CCAAT box [Currie, R.A. (1997) J. Biol Chem. 272, 30880–30888]. Using recombinant proteins, we confirm that at low concentrations of NF-Y, HMGI(Y) acts synergistically on the α2-collagen CCAAT and we extend this observation to HMGI and HMGI-C. However, enhancement of DNA binding to γ-globin, α-globin and MHC class II Ea CCAAT boxes was not observed. At high concentrations, HMGI proteins inhibit binding to α2-collagen and to γ-globin, but not to high affinity Ea or α-globin CCAAT. In none of our experiments did we see a ternary complex between NF-Y, HMGI(Y) and DNA. In protein competition experiments, NF-Y affinity was at least two orders of magnitude higher, even in the context of the suboptimal γ-globin CCAAT. Our data prove that HMGI proteins have complex positive and negative effects on NF binding to some, but not to all CCAAT boxes, suggesting that this phenomenon is dictated by the sequences flanking the pentanucleotide rather than direct protein-protein interactions.

Key words: NF-Y; HMG; CCAAT

1. Introduction

The CCAAT box is a widespread element present in a variety of eukaryotic promoters. Analysis of 502 unrelated promoters established that a high proportion (30%) contain the CCAAT pentanucleotide in either the forward or reverse orientation in the −60/−100 region, and identified highly preferred flanking sequences [1]. A survey of a database with 178 NF-Y binding sites showed that most eukaryotic CCAAT boxes are indeed recognized by this activator [2]. NF-Y is a ubiquitous heteromeric protein composed of three subunits, NF-YA, NF-YB and NF-YC, all necessary for DNA binding [3], whose genes have been cloned in several species (see [2] for review). A tight association between NF-YB and NF-YC is a prerequisite for NF-YA binding and sequence-specific DNA interactions [3] and their conserved domains contain putative histone fold motifs [4]. This motif, composed of three α-helices separated by short loops/strand regions, enables histones to dimerize with companion subunits, and to form the histone octamer [5]. NF-YA also contains a conserved domain, harboring separable NF-YB/NF-YC and DNA binding subdomains with no resemblance to known motifs [6].

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The high mobility group proteins HMGI-C, HMGI and HMGI(Y) are a family of abundant nuclear proteins associated with chromatin, that are thought to play important roles in such diverse processes as recombination and transcription (see [7] for review). They bind DNA in the minor groove, recognizing AT-rich sequences via short basic domains termed AT hooks [8]. While HMGI and HMGI(Y) are different splicing products of the same gene, and therefore are collectively named HMGI(Y), HMGI-C is encoded by a separate gene [9]. HMGI proteins were recently shown to affect gene expression by influencing the binding of transcription factors to their target DNA sequences: this effect is positive on some activators [10–15], but negative on homeodomain containing proteins [16,17]. NF-Y was among the transcription factors whose DNA binding and transcriptional potential was reported to be increased by HMGI(Y) [15]. To extend and better define this observation, we used recombinant NF-Y, HMGI-C, HMGI(Y), HMGI in electrophoretic mobility shift assays (EMSA) on several CCAAT boxes.

2. Materials and methods

2.1. Proteins production and purification

Murine HMGI cDNA was amplified and cloned in the bacterial expression vector pAR3038, as reported for the other two, HMGI(Y) and HMGI-C [17]. All HMGI proteins were expressed using the BL21(DE3) *Escherichia coli* strain, which contains the T7 RNA polymerase under the lacUV5 promoter control and purified by reverse-phase high performance liquid chromatography as previously described [17]. The consistency between purified recombinant HMGI proteins and calculated molecular masses from sequences was checked by mass spectrometry (Perkin-Elmer API 1). NF-YA and NF-YB proteins were produced from PET3b expression vectors in BL21(DE3), as inclusion bodies according to the protocols described previously [18]. NF-YC was produced from PET32b as a His-tagged fusion protein. BL21(DE3) Lys S bacteria were induced and inclusion bodies prepared, resuspended in 6 M GnCl, renatured by slow dialysis, together with equimolar amounts of recombinant NF-YB and NF-YA subunits and further purified on a NTA-agarose column (Qiagen). All cDNAs produced by PCR were checked by sequencing once they were introduced in the expression vectors [18].

2.2. Electrophoretic mobility shift assay (EMSA)

EMSA was performed with the indicated labelled oligonucleotides (10 000 cpm) incubated at 20°C for the indicated time in NF-Y binding buffer (50 mM KCl, 20 mM HEPES-HCl pH 7.9, 5 mM $MgCl_2$, 1 mM dTT, glycerol 3%), or in the conditions employed in [15] (100 mM NaCl, 10 mM Tris-HCl pH 7.5, 1 mM dTT, 1 mM EDTA, 5% glycerol). Samples were loaded on a 4.5% acrylamide 0.5×TBE gel and run at 4°C for 3 h at 200 V. Gels were dried on 3MM paper and exposed.

3. Results

The recent report showing that HMGI(Y) increases the affinity of NF-Y for the α2-collagen CCAAT [15] prompted

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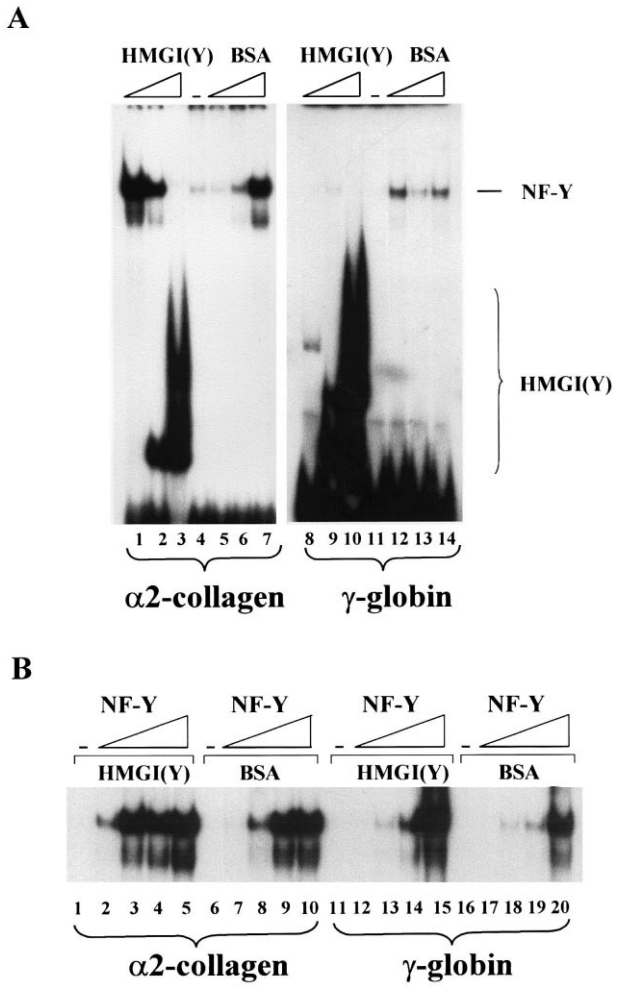

Fig. 1. NF-Y and HMGI proteins binding to the α2-collagen and γ-globin CCAAT boxes. A: EMSA dose response (1, 10, 100 ng) of HMGI(Y) on the α2-collagen (lanes 1–7) and γ-globin (lanes 8–14) CCAAT boxes, in the presence of a constant amount of NF-Y (0.05 ng). NF-Y incubated alone (lanes 4, 11), with increasing amounts (1 ng in lanes 1, 8; 10 ng in lanes 2, 9; 100 ng in lanes 3, 10) of HMGY (lanes 1–3 and 8–10), and of control BSA (lanes 5–7 and 12–14). B: EMSA dose response of NF-Y (0.05, 0.2, 0.6, 2 ng) on the α2-collagen (lanes 1–10) and γ-globin (lanes 11–20) CCAAT boxes in the presence of a constant amount of HMGI(Y) (1 ng). Lanes 1 and 11: HMGI(Y) alone; lanes 6 and 16: BSA alone.

us to investigate its effect on the proximal γ-globin CCAAT which we have recently shown to be targeted by NF-Y [19]. This site contains a sequence overlapping the CCAAT box (CTTGACCAATAGTCTT) potentially interacting with HMGI proteins, which are known to require four ATs for efficient DNA binding [7]. NF-Y and HMGI proteins were produced in bacteria and purified; HMGI proteins were checked for purity and integrity by mass spectrometry. We employed the α2-collagen oligonucleotide used by Currie [15] and another one containing the proximal human γ-globin CCAAT box in parallel EMSA experiments; the dose response of HMGI(Y) on a fixed, low (0.05 ng) amount of NF-Y is shown in Fig. 1A: without HMGI(Y), the amount of bound NF-Y was barely detectable on α2-collagen (lane 4) and not visible on γ-globin (lane 11). When low doses (1, 10 ng) of HMGI(Y) were added, a dramatic positive effect was seen on α2-collagen, as compared to the control BSA (compare lanes 1, 2 with 4–6); on γ-globin this effect was not visible compared with the BSA control (compare lanes 8, 9 with 11–14). Consistent with the presence of four ATs, the γ-globin site yielded a strong HMGI(Y) band at lower concentrations of HMGI(Y). At higher doses (100 ng) of HMGI(Y) the NF-Y band was abolished on the α2-collagen and a very intense faster migrating HMGI(Y) complex was predominant (Fig. 1A, compare lanes 3 and 4–7). We performed the reverse experiment, fixing the amount of HMGI(Y) at 1 ng, corresponding to the dose of maximal cooperative effect, and increasing the concentrations of NF-Y; Fig. 1B shows that clear synergy is visible at low doses of NF-Y only on α2-collagen, as compared with addition of control BSA (0.05–0.2 ng, compare lanes 2 and 3 with 7 and 8, respectively). At higher NF-Y doses and on γ-globin, the effect was not evident (Fig. 1B, compare lanes 4, 5 with 9, 10; lanes 12–15 with 17–20). NF-Y binding in these experiments was performed in the buffer indicated by Currie, which contains no $MgCl_2$, and was also confirmed in the binding conditions we routinely use [18,19] that contain 5 mM $MgCl_2$ (not shown). With respect to the cited study, (i) we observed a similar positive effect of HMGI(Y), albeit at lower doses, 1 and 10 ng vs. 10–25 ng, on suboptimal amounts of NF-Y; this behavior is specific for α2-collagen and absent on the γ-globin CCAAT. (ii) As in Currie's study, a complex of altered mobility corresponding to simultaneous binding of HMGI proteins and NF-Y could not be detected under our binding and electrophoretic conditions.

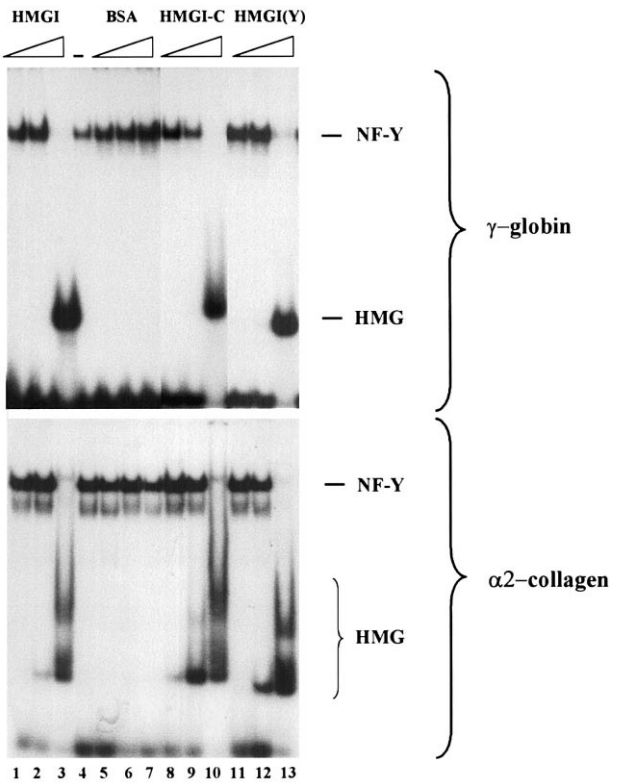

Fig. 2. Negative effect of HMGI proteins at high NF-Y concentrations. The α2-collagen and γ-globin CCAAT oligonucleotides were incubated with 0.5 ng of NF-Y either alone (lane 4), with increasing amounts (1, 10, 100 ng) of HMGI(Y) (lanes 11–13), HMGI (lanes 1–3) and HMGI-C (lanes 8–10), or control BSA (lanes 5–7).

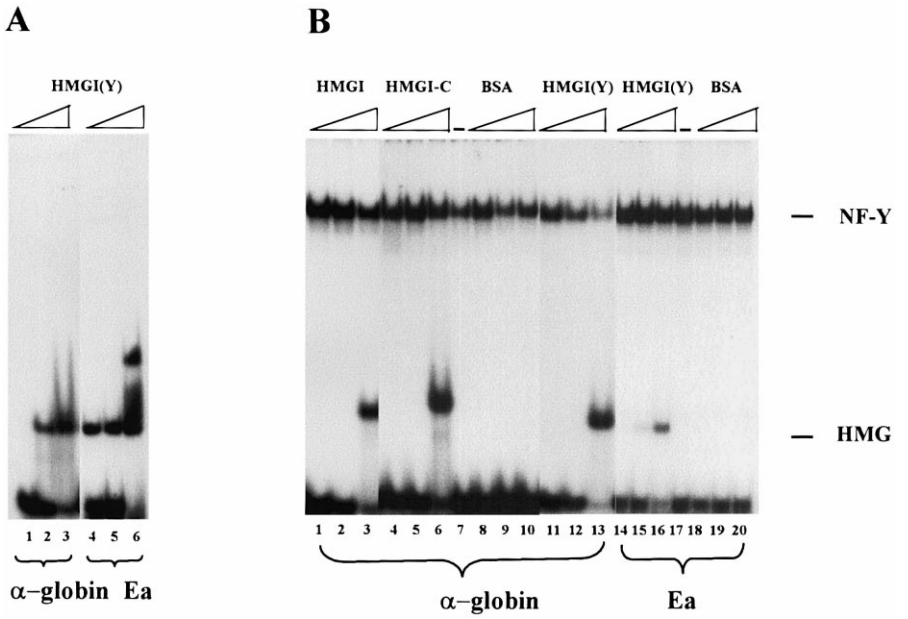

Fig. 3. NF-Y and HMGI proteins binding to the α-globin and MHC class II Ea Y-box. A: EMSA dose response (1, 10, 100 ng) of HMGI(Y) on α-globin (lanes 1–3) and Ea CCAAT (lanes 4–6). B: Same as A, except that HMGI(Y) was incubated together with 0.2 ng of NF-Y on the α-globin (lanes 1–13) and Ea Y-box (lanes 14–20). NF-Y incubated alone (lanes 7 and 17), with 1, 10, 100 ng of BSA (lanes 8–10, and 18–20, respectively). In lanes 1–6 equivalent amounts of HMGI and HMGI-C were used.

The previous experiments performed at low doses of NF-Y also raised the possibility that HMGI(Y) might have a negative effect on CCAAT binding. We therefore wished to complete our observations with a higher amount of NF-Y (0.5 ng) and extend them to the other HMGI and HMG-C proteins. Fig. 2 shows that at 100 ng, HMGI(Y) almost completely inhibited NF-Y binding both to α2-collagen and to γ-globin CCAAT boxes (Fig. 2, lanes 3 and 4). As expected, the control BSA provoked little modification in the intensities of the NF-Y bands. The negative effect was equally evident with comparable high amounts of HMGI and HMGI-C and with both oligonucleotides (Fig. 2, lanes 10 and 13). Again, no slower tertiary complex was visible at these higher NF-Y concentrations. We conclude that binding of HMGI proteins is detrimental to NF-Y-CCAAT interactions, possibly because of a mutually exclusive DNA interaction.

Both α2-collagen and γ-globin CCAAT boxes are mid-range in terms of affinity for NF-Y [19–21]; we wished to test the effect of HMGI(Y) on high affinity NF-Y sites. The α-globin and MHC class II Ea Y-box represent ideal candidates, since they are both among the strongest sites (K_d 5×10^{-11} [20,21]) yet they contain very different flanking sequences: the former is embedded in a GC-rich sequence (GCCGACCAATGAGCGC), devoid of good HMGI(Y) binding sites, while Ea is surrounded by runs of ATs (TTTTAACCAATCAGAAAAAT) that represent candidate sites for HMGI proteins. Firstly, we tested in EMSA the affinity of HMGI(Y) for these oligonucleotides: as expected, the dose-response experiment shown in Fig. 3A indicates that a nucleoprotein complex is already visible at 1 ng of HMGI(Y) on the Ea oligonucleotide, and multiple bands are present at 100 ng (Fig. 3A, lanes 4–6); on α-globin, affinity appears to be at least one order of magnitude lower (compare lanes 1–3 with 4–6). Similar results were obtained with the other HMGI proteins (data not shown). Using the labelled α-CCAAT oligonucleotide, recombinant NF-Y was pre-incubated under conditions (0.5 ng) in which 50% of the labelled DNA was complexed with NF-Y, either alone (Fig. 3B, lane

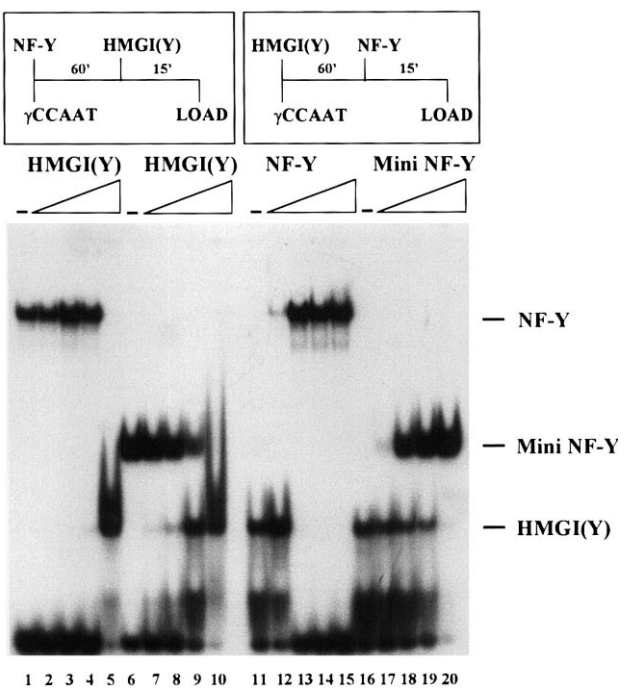

Fig. 4. NF-Y and HMGY binding affinities on the γ-globin CCAAT box. NF-Y (0.5 ng, lanes 1–5) or HAP homology mini-NF-Y (0.5 ng, lanes 6–10) were preincubated with the labelled γ-globin CCAAT oligonucleotide and then 0.1, 1, 10, 100 ng of HMGI(Y) was added. In lanes 11–20, 10 ng of HMGI(Y) was preincubated with the labelled oligo before addition of 0.1, 1, 10 and 100 ng of NF-Y (lanes 12–15) or mini-NF-Y (lanes 17–20).

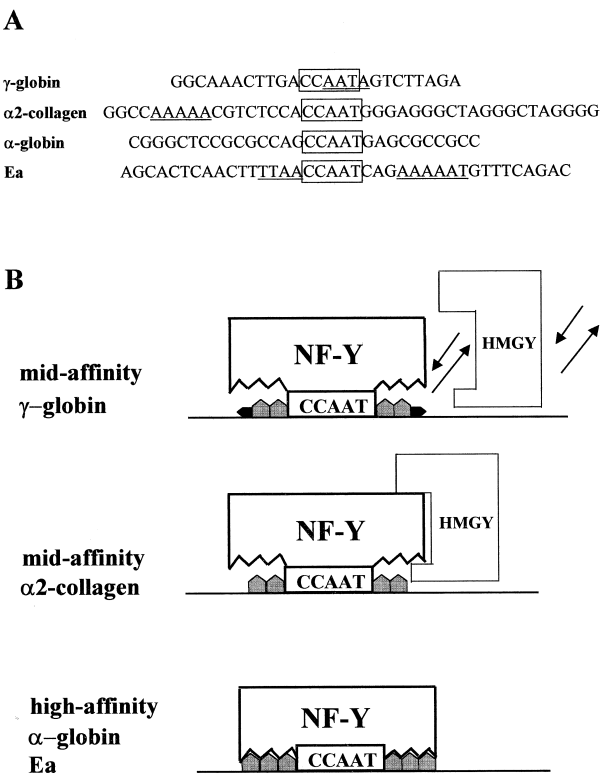

Fig. 5. Scheme of NF-Y/HMGI(Y) interactions on the different sites. A: Sequences of the oligonucleotides used in this study; potential HMGI binding sites are underlined. B: Models for NF-Y/HMGI(Y) interactions on the three types of sites identified in this study.

7), together with increasing concentrations (1, 10, 100 ng) of HMGI(Y) or with BSA control (lanes 11–13 and 8–10, respectively): NF-Y binding was not substantially modified by either incubation, but a small decrease was observed at 100 ng concentration, when binding of the HMGI(Y) protein became visible. We performed parallel experiments with the other HMGI proteins and the pattern was very similar (Fig. 3B, lanes 1–6). Note that the binding of HMGI(Y) to its target sites was severely inhibited: 100-fold more protein was necessary to obtain the same degree of HMGI(Y) binding (compare lanes 2 in Fig. 3A and 2, 5, 12 in Fig. 3B). In parallel, we used the Ea Y box oligo: no decrease in NF-Y binding was essentially visible, while the inhibition of HMGI(Y) single and multiple binding was extremely pronounced (compare in Fig. 3A lanes 4–6 and in Fig. 3B, lanes 14–16). As for the previous CCAAT boxes, no additional complex slower (or faster) was detected on these sites. Note that unlike α2-collagen, on α-globin and Ea CCAAT, low doses of HMGI proteins had no effect on low doses of NF-Y, a situation that parallels the results with γ-globin (data not shown). These data strongly suggest that binding of the two proteins is not cooperative on these sites and indeed mutually exclusive.

To better define the relative affinities of NF-Y and HMGI proteins for overlapping sites, we performed EMSA experiments changing the order of addition. Moreover, since the slow electrophoretic mobility of wt NF-Y in our gel system might theoretically preclude the visualization of a ternary complex, we also used an NF-Y mutant containing only the evolutionarily conserved domains representing the minimal DNA binding parts of NF-Y [18]: the migration of the mini-NF-Y is much faster and could allow the visualization of such double binding. For these experiments, we used the γ-globin site (best for HMGI proteins and worst for NF-Y in terms of affinity) with HMGI(Y), the HMGI protein that showed the highest affinity for DNA in our assays. We preincubated 0.5 ng of either wt NF-Y (Fig. 4, lanes 1–5), or the mini-NF-Y (Fig. 4, lanes 6–10) for 60 min, then we added increasing concentrations of HMGI(Y) (0.1, 1, 10, 100 ng) and pursued incubation for further 15 min. HMGY was able to displace wt NF-Y binding only at 100 ng, whereas for the mini-NF-Y 10 ng was sufficient to give a 50% inhibition (compare lanes 5 and 9 in Fig. 4). The opposite experiment was also performed, namely preincubation of 10 ng of HMGI(Y) and later addition of increasing (0.1, 1, 10, 100 ng) amounts of wt or mini-NF-Y (Fig. 4, lanes 11–15 and 16–20, respectively): with the wt NF-Y protein, complete inhibition of HMGI(Y) binding was seen already at 1 ng of NF-Y, while higher concentrations were necessary for the mini-NF-Y, although 1 ng already yielded an NF-Y band that was more intense than that of HMGI(Y) (Fig. 4, compares lanes 11–13 and lanes 16–20). We conclude that even within this suboptimal NF-Y binding site, the affinity of HMGI(Y) for its target sequence is two orders of magnitude lower than that of NF-Y; the difference with the conserved domains NF-Y mutant is somewhat lower, but still very significant.

4. Discussion

In this report we investigated the binding of the CCAAT transcription factor NF-Y and HMGI proteins to overlapping DNA sequences. A previous study indicated that (i) NF-Y binding to the α2-collagen CCAAT box is highly facilitated by addition of HMGI(Y); (ii) overexpression of HMGI(Y) in transfection assays modestly increases a promoter containing multimerized CCAAT boxes (2.5-fold) and (iii) HMGI(Y) interacts in solution with the C-terminal half of NF-YA [15]. Indeed we found that significant facilitation of NF-Y binding occurs in the presence of all HMGI proteins on the α2-collagen site when low amounts of HMGI and NF-Y are used, but observed no effect on α- and γ-globin and Ea CCAAT boxes at high NF-Y concentrations. On the other hand, high concentrations of HMGI proteins are inhibitory of NF-Y binding on the α2-collagen and γ-globin, but not on the α-globin and Ea CCAAT.

Several lines of evidence indicate that NF-Y plays a pivotal role in the activation of a wide variety of gene promoters. Biochemical experiments have proved that NF-Y is among the best DNA binders in terms of efficiency (in the pM range) and, despite the histone-like nature of NF-YB and NF-YC, in terms of specificity as well, being able to discriminate nucleotides over one turn of the double helix with the central CCAAT as an essential core [2,20,21]. NF-Y bends and twists DNA in a fashion that is highly reminiscent of histone-DNA interactions [19,22]. Functional experiments in different systems have clearly established the importance of NF-Y binding CCAAT boxes. In vitro transcription experiments suggested that NF-Y is involved in the early phases of pre-initiation complex formation and in re-initiation. NF-Y can help other transcription factors (Sp1, RFX, SREBP2) bind to their target sites and facilitates c/EBP transcriptional activation on the albumin promoter [2]. Footprinting analysis invariably finds NF-Y binding sites of active promoters protected. Because of

all these features and its highly preferred position within promoters, NF-Y might play an architectural role in gene activation. HMG proteins in general, and HMGI proteins in particular, are also thought to be architectural factors connecting gene-specific activators and chromatin structures. Efforts aimed at elucidating the effect of HMGI on transcription factor binding and activation lead to a wide spectrum of results: several promoters (TNF-β, IFN-β, IL2Rα, E-selectin) are up-regulated by cotransfections with HMGI expression vectors, while IL4 and GP91-phox are inhibited [7]. Interestingly, none of the former contain CCAAT boxes, while IL4 and GP91-phox harbor multiple NF-Y binding sites that are crucial for transcriptional regulation [23,24]. The role of the abundant chromatin-associated HMGI proteins might thus be positive or negative, depending on the promoter context and the architecture of the single activator binding sites. Consistent with these observations, HMGI proteins were shown to synergize or antagonize different transcription factors: ATF-2 and NF-κB binding and activation are positively modulated by flanking HMGI AT-rich sites [10,11,14], while binding and activation of homeobox proteins are inhibited by HMGI-DNA interactions with overlapping sites [17]. We note that NF-Y, like homeobox proteins, interacts with both the major and the minor groove [22]. Methylation interference, IC substitutions and binding inhibition with MGBs (minor groove binding drugs) indicate that NF-Y indeed contacts the minor groove in the AAT sequence, which is most likely the target of HMG-I proteins on the γ-CCAAT box oligo used here (see Fig. 5). It is not clear at the moment whether synergy is due to protein-protein interactions, direct binding to DNA, or indirect facilitation of DNA binding. We do not favor the first hypothesis, despite evidence that HMGI(Y) can interact with NF-YA in solution, since in that case we would have expected to see an effect on all CCAAT boxes tested. Rather, an important conclusion of the present study is that even within the same factor (NFY) the effect of HMGI proteins depends on the fine molecular structure of the target DNA site: in general, our results indicate that the NF-Y/HMGI interactions are more complex than previously thought [15]. The effect is (i) synergistic on CCAAT boxes lacking an overlapping AT stretch, such as α2-collagen, whose intermediate affinity is improved by HMGI proteins; (ii) null, even at high concentrations, on stronger sites, such as α-globin and Ea Y-box; (iii) negative (at high concentrations) on sites containing an A or T after the central CCAAT, such as γ-globin (see Fig. 5). Note that the central CCAAT pentanucleotide is identical in all NF-Y target sequences: the differential effects must therefore be due to the flanking sequences, which are known to dramatically influence NF-Y affinity [2,21,25]. It is possible that a specific subset of CCAAT boxes evolved the capacity to coexist with HMGI sites; this is also supported by the observation that 20% of all CCAAT boxes statistically contain an A or T at the 3′ [2], nucleotides determining a decrease in NF-Y affinity [21,25]. Even on sites such as the γ-globin, however, our protein competition experiments indicate that NF-Y has a 2 log advantage in terms of affinity for a CCAAT sequence, and probably more for an optimal CCAAT such as the Y box. This could in theory be functionally compensated by the physiologically higher concentrations of HMGI proteins in the nucleus [7]. Whether it is indeed so, is a matter of further investigation.

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