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High Mobility Group I Proteins Interfere with the Homeodomains Binding to DNA*

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Homeodomains (HDs) constitute the DNA binding domain of several transcription factors that control cell differentiation and development in a wide variety of organisms. Most HDs recognize sequences that contain a 5′-TAAT-3′ core motif. However, the DNA binding specificity of HD-containing proteins does not solely determine their biological effects, and other molecular mechanisms should be responsible for their ultimate functional activity. Interference by other factors in the HD/DNA interaction could be one of the processes by which HD-containing proteins achieve the functional complexity required for their effects on the expression of target genes.

Using gel-retardation assay, we demonstrate that two members of the high mobility group I (HMGI) family of nuclear proteins (HMGI-C and HMGY) can bind to a subset of HD target sequences and inhibit HDs from binding to the same sequences. The inhibition of the HD/DNA interaction occurs while incubating HMGI-C with DNA either before or after the addition of the HD.

The reduced half-life of the HD·DNA complex in the presence of HMGI-C, and the shift observed in the CD spectra recorded upon HMGI-C binding to DNA, strongly suggest that structural modifications of the DNA are responsible for the inhibition of the HD·DNA complex formation. Moreover, by co-transfection experiments we provide evidence that this inhibition can occur also *in vivo*.

The data reported here would suggest that HMGI proteins may be potential regulators of the function of HD-containing proteins and that they are able to interfere with the access of the HD to their target genes.

Homeodomains (HDs)[1] are 61-amino acid-long structures that are able to interact with DNA in a sequence-specific manner (1). They represent the DNA binding domain of a large number of transcription factors that control cell fate decisions in a wide range of organisms, including yeast, insects, and vertebrates (2, 3). The structure of HDs and their mode of DNA interaction are conserved (1, 4, 5). With a few exceptions, sequences recognized by HDs possess a 5′-TAAT-3′ core motif (6–8). Though recognizing similar DNA sequences, when expressed in the same temporal and spatial context, distinct HD-containing proteins may show different biological activities (9, 10). These findings indicate that the DNA binding specificity of these proteins cannot be the only molecular event that determines their biological effect. In fact, some other molecular mechanisms have been identified, in addition to the DNA binding specificity, that allow the selection of different target genes by distinct HD-containing proteins (11, 12). One of these mechanisms is based on the differential interaction of HD-containing proteins with accessory factors that mediate the contacts with the basal transcriptional machinery. For example, the Pou-domain-containing proteins Oct-1 and Oct-2 bind to the same DNA sequence, yet only the former is able to interact with the acidic transcriptional activator VP16 (11). Another mechanism is based on specific protein-protein interactions with other factors that bind to DNA tracts located near the sequences recognized by an HD-containing protein. A clear example of this phenomenon is the interaction between Ultrabithorax and Extradenticle proteins (12).

A simple molecular mechanism to control the binding of HD-containing transcription factors to a DNA regulatory element could be the presence of competitors for the interacting DNA sequence. In this paper we show that the high mobility group I proteins (HMGI, HMGY, and HMGI-C) may have this function. The HMGI proteins are a family of low molecular mass non-histone nuclear proteins rich in both basic and acidic residues, which comprise an important component of the active chromatin structure (reviewed in Ref. 13) and that have been shown to bind in the minor groove of AT-rich sequences of DNA through a DNA binding motif called AT-hook (14–17). This family includes three polypeptides: HMGI and HMGY derived from alternative splicing of the same gene and differing from each other by 11 amino acids (18) and a third protein called HMGI-C that is the product of a separate gene (19, 20).

HMGI(Y) have been demonstrated to contribute to the transcriptional regulation of several promoters by interacting with different transcription factors (21–27). The human interferon β gene provides a particularly well characterized example of how HMGI(Y) could interact with specific DNA sequences on the promoter and cooperate with other proteins such as ATF-2, NF-κB, and c-Jun in activating this gene by stimulating the binding of these transcription factors to their target sites (28, 29).

An elevated expression of all these proteins has been correlated with malignant phenotypes in epithelial and fibroblastic rat thyroid cells, and in many experimental carcinomas (30–32). In addition, at least one of them, HMGI-C, has been found

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[1] The abbreviation used are: HD(s), homeodomain(s); HMG, high mobility group; En, Engrailed; GST, glutathione *S*-transferase.

to be important for the retrovirally induced transformation of rat thyroid cells (33) and many groups have recently reported a correlation between rearrangements of the HMGI-C gene and some benign human mesenchymal tumors (34, 35). Furthermore, the inactivation of the *Hmgi-c* gene has been demonstrated to cause the pygmy phenotype in the mouse (36), postulating an important role of this protein in cell proliferation and differentiation.

The DNA binding specificity of HMGI proteins (preference for AT-rich sequences) would suggest that these molecules may interact with the 5′-TAAT-3′-containing sequences, specific HDs target sequences. In this way, HMGI proteins might interfere with the DNA binding function of HD-containing transcription factors and therefore influence the biological effect of these proteins.

In this study, we demonstrate that HMGI proteins are indeed able to interact with some HD binding sequences. Specifically, we show that the binding of HDs and HMGI proteins to 5′-TAAT-3′ motif-containing sequences is mutually exclusive. In particular, the presence of HMGI proteins blocks HDs from binding to 5′-TAAT-3′ sequences. Apparently this effect is mediated by structural modifications of the DNA induced by HMGI proteins and not only by a simple competition for the same target sequence. As a model to study the functional role of these interactions, we utilize the transcriptional activation of HOXD9 on an autoregulatory element (37). We show that HMGY is able to compete for the binding of HOXD9 to the DNA *in vitro* and to reduce the target-specific transcriptional activation of HOXD9 in transfected cells.

EXPERIMENTAL PROCEDURES

Oligonucleotides—The following oligonucleotides were used in gel-retardation assay experiments (only upper strand sequence shown): C_{Ant1}, 5′-CACTGCCCAGTTAATTGTTCTTGA-3′; PRDII, 5′-GGGAAATTCCGTGGGAAATTCCGAGCT-3′; NP, 5′-TGATATTTAATTGATTTT-3′; C_{Ant4}, 5′-CACTGCCCAGTTAAGTGTTCTTGA-3′; T109, 5′-GCACGCTAATTGAGACGC-3′; HCRII, 5′-GACACATTAATCTATAATCAAATAC-3′

Protein Expression and Purification—The plasmid coding for Engrailed (En) has been already described (38). In this plasmid the transcription of the HD-coding sequence is driven by the T7 RNA polymerase promoter. The murine HMGI-C and HMGY coding sequences were amplified by polymerase chain reaction using the appropriate primers deduced from the amino and carboxyl termini, and cloned between the *Nde*I and *Bam*HI sites of the bacterial expression vector pAR3038 under the bacteriophage T7 promoter (39). The resulting clones were verified by sequencing. All proteins were expressed using the BL21 (DE3) *Escherichia coli* strain, which contains the T7 RNA polymerase under lacUV5 promoter control (39). Cultures were grown to A_{600} = 0.6, induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside, grown overnight, and harvested.

EnHD used for DNA-binding assays was partially purified by using Econo Pac S cartridges (Bio-Rad). The concentration of the active protein was measured by oligonucleotide saturation assay. A gel-retardation assay (see below) was performed with increasing amounts of oligonucleotides (from 0.3 to 50 nM), then the protein-bound and free oligonucleotide concentration values were subjected to a Scatchard plot analysis. The value of the active protein percentage over the total amount was usually 40–80%.

HMGI proteins were selectively extracted from bacterial cells with 5% (v/v) perchloric acid and precipitated with acetone-HCl as described previously (30). The proteins were purified by reverse-phase high performance liquid chromatography on a Bio-Rad RP304 column using a Waters apparatus as elsewhere described (32). The consistency between purified recombinant HMGI proteins and calculated molecular mass from sequence was checked by mass spectrometry (Perkin-Elmer API 1 spectrometer). The construct encoding GST-HOXD9 was described previously (40), and the GST fusion protein was expressed and purified according to standard procedures (41).

Gel-retardation Assay and Quantitation of the Binding Activity—Double-stranded oligonucleotides were labeled at the 5′-end with ^{32}P and used as probes in the gel-retardation assays. Gel-retardation assay was performed incubating protein and DNA in a buffer containing 20 mM Tris-HCl, pH 7.6, 75 mM KCl, 55 μg/ml bovine serum albumin, 3 mM dithiothreitol, 13% glycerol for 30 min at room temperature. Protein-bound DNA and free DNA were separated on native 7.5% polyacrylamide gel run in 0.5 × TBE (Tris borate-EDTA), 300 V at 4 °C. Gels were dried, exposed to x-ray films, and bands were quantitated by densitometric scanning of the autoradiogram using a LKB laser densitometer. The monoclonal antibody against GST protein was purchased from Santa Cruz Biotechnology and used at a final concentration of 10 μg/ml.

Circular Dichroism and Structure Analysis—Jacketed cells of 2–5 mm were used, and typically 10 spectra were accumulated, averaged, and base line-corrected with a spectrum of buffer alone on a Jasco J-600 spectropolarimeter interfaced to an Olidata personal computer. Calibration of the instrument was performed with D(+)-10-camphorsulfonic acid at 290 nm. All spectra were collected at 10 ± 0.1 °C. Thermostability was controlled by a Haake F3 water bath. Spectra were recorded in 10 mM sodium phosphate buffer, pH 7.5, KCl 50 mM.

Cell Culture and Transfection—The expression construct producing HOXD9, as well as pT81luc and pTHCR reporter constructs, were described elsewhere (40). The pSVHMGY expression construct was obtained subcloning a 1.7-kilobase pair *Eco*RI fragment containing the full-length murine HMGY cDNA (18) into the polylinker of pGDSV7 expression vector (42). NIH-3T3 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin, at 37 °C in a humidified 5% CO_2 incubator. Cells were transfected by the calcium phosphate co-precipitation procedure (43). Briefly, cells were plated at a density of 0.5 × 10^6/60-mm diameter culture dish. Precipitates, containing 1 μg of reporter plasmid, 7 μg of expression constructs, and 0.5 μg of pTK β-galactosidase expression vector as internal control, were applied to subconfluent cells for 16 h before changing medium. Total amounts of each plasmid were kept constant in all experiments, by adding the corresponding empty vectors.

β-Galactosidase and Luciferase Assays—48 h after transfection cells were harvested and lysed with Promega's lysis buffer. Luciferase enzyme assays were performed using the luciferase assay system (Promega) in a luminometer (Lumat LB 9501, EG & G Berthold) according to manufacturer's instructions; fluorimetric β-galactosidase assays were performed using 4-methylumbelliferyl-β-D-galactoside (MUG-Sigma) as substrate (44). Luciferase activity of the reporter vectors was then normalized for transfection efficiency using β-galactosidase activity.

RESULTS

HMGI Proteins Recognize HD Binding Sequences—To test whether HMGI proteins are able to interact with sequences recognized by HDs, the binding activity of recombinant HMGI-C and HMGY to several oligonucleotides containing the 5′-TAAT-3′ motif was evaluated. Results are shown in Fig. 1. The oligonucleotide PRDII contains two copies of the human interferon β promoter HMGI(Y) binding sequence (28) and therefore provided a positive control. Both HMGI-C and HMGY efficiently recognize the oligonucleotide CAnt1, which is a dominant interaction site for both Antennapedia and EnHDs (45). The binding activity of both proteins requires the integrity of the 5′-TAAT-3′ motif. In fact, the oligonucleotide CAnt4, in which the 5′-TAAT-3′ motif has been changed to 5′-TAAG-3′, is no longer bound by either HMGI-C or HMGY. Both proteins are also able to interact with the NP oligonucleotide, which is a binding site for several HD-containing proteins (Ref. 46 and references therein). HMGI proteins may recognize only a subset of HDs binding sequences. In fact, neither HMGI-C nor HMGY are able to establish an efficient interaction with the oligonucleotide T109, which is recognized by both Antennapedia and EnHDs (data not shown).

Interference between HDs and HMGI Proteins in DNA Binding—The evidence that HMGI proteins interact with some HD binding sequences would predict that this class of proteins and HDs may influence each other during the interaction to common sites. We have evaluated this possibility incubating together HDs and HMGI proteins with CAnt1 oligonucleotide. Results are shown in Fig. 2. The presence of either HMGI-C or HMGY completely blocks the binding of EnHD.

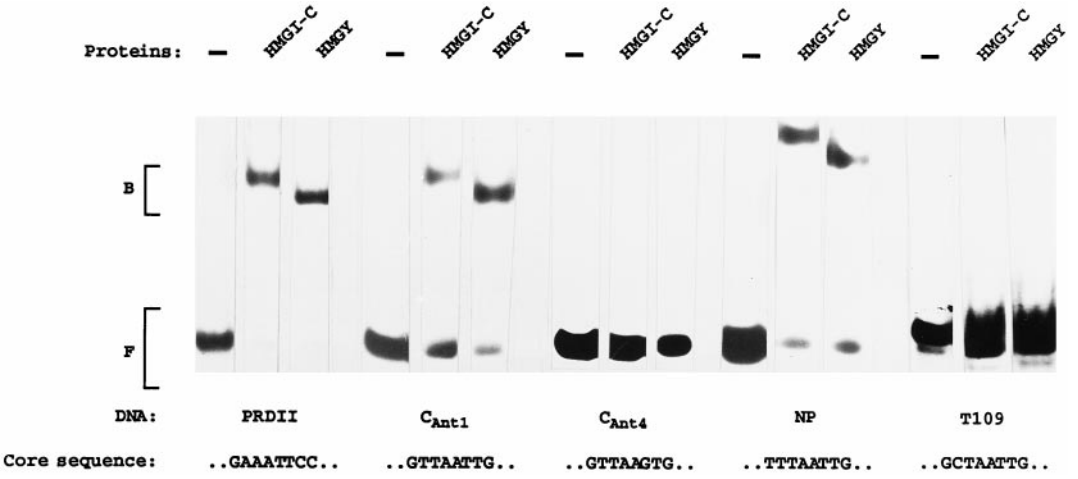

FIG. 1. **HMGI proteins recognize HD binding sites.** The full oligonucleotide sequences are shown under "Experimental Procedures." Here only the putative HMGI/HD binding sequence is shown. Oligonucleotides were labeled at the 5′-end with the same specific activity and used at a final concentration of 0.05 μM. Both HMGY and HMGI-C proteins were used at the final concentration of 0.6 μM. *F*, free DNA; *B*, protein bound to DNA.

It has been demonstrated recently that HMG1, another member of the HMG family, is able to interact with HOX proteins in absence of DNA binding (40). Therefore, we have tested whether the effect of HMGI proteins on HD binding requires HMGI/DNA interaction. As shown in Fig. 3, HMGI-C is able to prevent the EnHD/CAnt1, but not the EnHD/T109 interaction. These data, along with our experimental observation that HMGI-C is not able to efficiently recognize the T109 oligonucleotide (see Fig. 1), indicate that the HMGI/DNA interaction is necessary to block the EnHD binding to DNA. These results indicate that the effect of HMGI proteins on HD/DNA binding is not due to direct protein-protein interactions. It is important to note that the inhibitory effect is observed at a HMGI-C protein/EnHD molar ratio of 1.8. Since HMGI proteins are present in the cell in large amounts (47), the HMGI/EnHD molar ratio used should be lower than in the nucleus. This estimation suggests that the interference that we observe may occur *in vivo*.

Mechanism by Which HMGI Proteins Inhibit HD Binding to DNA—HDs contact DNA both in the major groove (by the recognition helix) and in the minor groove (by the NH_2-terminal arm). The inhibitory effect of HMGI proteins, which interact with DNA only by minor groove contacts, could occur through a complex mechanism comprised of two molecular events. First, HMGI proteins gain access to DNA competing with the HDs NH_2-terminal arm for the establishment of contacts in minor groove. Subsequently, the interaction of HMGI proteins modifies the DNA structure so that the HD/major groove interaction is less efficient, leading to the release of the whole HD from the DNA. The structural modifications of DNA which occur upon interaction with HMGI proteins could support the existence of the second mechanism (48–50). To test this hypothesis several experiments were performed. The gel, shown in *A* of Fig. 4, demonstrates that the inhibitory effect of HMGI-C on the EnHD binding to CAnt1 occurs with the same efficiency when HMGI-C is added before or after the formation of the EnHD·DNA complex. Kinetic experiments revealed that HMGI-C inhibits EnHD binding in less than 10 s (data not shown). *Panel B* of Fig. 4 shows that when HMGI-C protein is used at a concentration in which the EnHD·CAnt1 oligonucleotide complex is still detectable, the half-life of the EnHD·CAnt1 complex is greatly reduced relative to that observed in the absence of HMGI-C protein. These data are compatible with a mechanism in which HMGI-C binds to the minor groove more efficiently than the NH_2-terminal arm of EnHD, which is repelled by the presence of HMGI-C. Consequently, we hypothesize that the HMGI-C binding to the minor groove influences the structure of the major groove in a way that EnHD is no

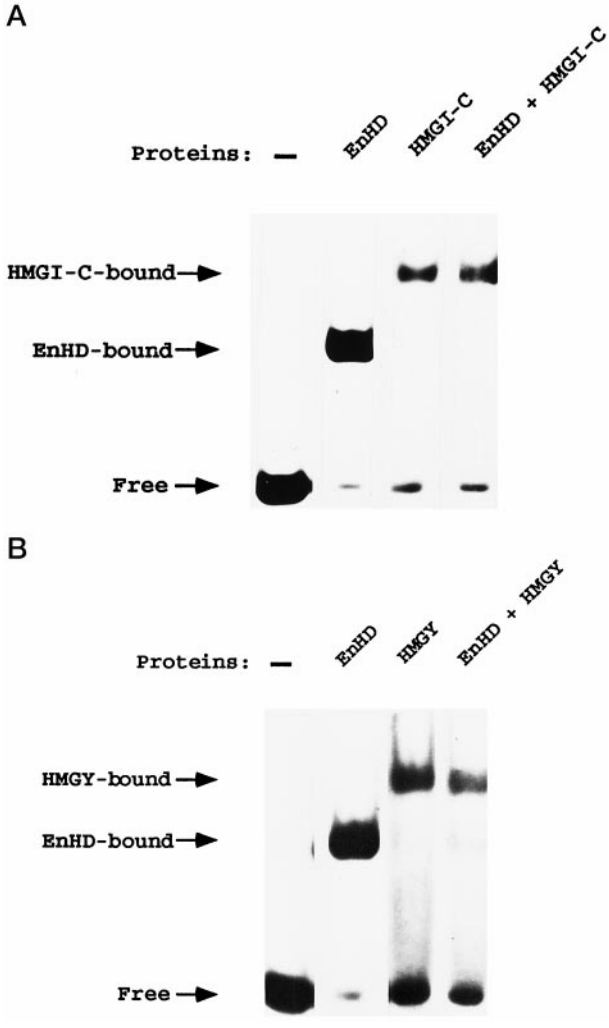

FIG. 2. **The presence of HMGI proteins inhibits the binding of EnHD.** The gel-retardation assay was performed as described under "Experimental Procedures." Proteins were used at the following final concentrations: HMGI-C, 1.1 μM; HMGY, 1.1 μM; EnHD, 0.6 μM. *A*, effect of HMGI-C; *B*, effect of HMGY.

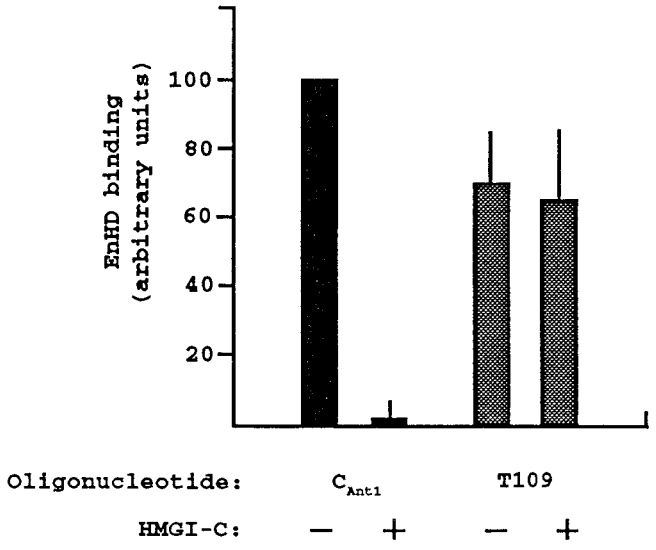

FIG. 3. **The HMGI-C-DNA interaction is required for the inhibitory effect on HD-binding.** Experimental conditions were those used in the experiments shown in Fig. 2. The values of EnHD binding to DNA have been obtained by densitometric scanning of gel-retardation assay autoradiograms. *Columns* and *bars* represent the mean values and the standard deviations, respectively, of three independent experiments.

longer able to bind to the DNA.

The drug berenil (Sigma) is able to interact with DNA into the minor groove but is not able to induce structural modifications on the bound DNA (51). Moreover, it has been demonstrated that, even at very high concentrations, this drug is not able to affect HDs/DNA interactions (52). Therefore, berenil should compete with HMGI-C for the minor groove interaction and reduce the inhibitory effect of this protein on HD binding to DNA. The effect of berenil on the inhibition of EnHD binding to DNA by HMGI-C protein is shown in Fig. 5. When used at a concentration of 800 μM, berenil determines only a modest reduction of EnHD binding to the CAnt1 sequence (compare *lanes 2* and *4*). However, the abolition of EnHD-binding by HMGI-C protein is significantly reduced by the presence of berenil (compare *lanes 3* and *5*). These results indicate that the establishment of a minor groove interaction without modification of the DNA structure (such as what occurs with berenil treatment) is not able to prevent HDs from binding to DNA, supporting the model that structural changes of DNA mediate the inhibitory effect of HMGI proteins on HD-DNA interaction.

To verify the structural modifications of DNA that could occur as a result of interaction with HMGI proteins, CD spectra of the HMGI-C·CAnt1 complex were recorded and compared with those of the CAnt1 oligonucleotide alone. Results are shown in Fig. 6. Following the interaction with HMGI-C the long-wavelength positive CD band of DNA decreases with a shift in the 258 nm crossover to longer wavelength. These modifications are likely due to significant structural alterations of the CAnt1 oligonucleotide, since the CD spectrum of the protein shows no bands of absorption in the region above 240 nm (data not shown). Similar CD changes have been observed in compaction of the DNA into particles *in vitro*, into phage heads or into nucleosomes (53–55). While we have demonstrated that structural modifications of the CAnt1 sequence occur upon HMGI-C binding, a more complete understanding of the reaction mechanism involved will necessitate higher resolution structural studies. Nevertheless, our CD data are compatible with the binding data shown in Figs. 4 and 5, suggesting that part of the HMGI-C inhibition of the EnHD-CAnt1 interaction is due to a structural modification of the DNA.

HMGY Is Able to Inhibit in Vivo HOXD9-mediated Transcriptional Activation—To test whether the inhibitory effect of HMGI proteins on the HD-DNA interaction could have an *in vivo* relevance, we measured the effect of expression of HMGY on transcriptional activation induced by a HOX protein by co-transfection assay. As reported by Zappavigna *et al.* (37), the *HOXD9* gene product activates transcription binding to an autoregulatory element (HCR) deriving from its own promoter. The ~100-base pair HCR element contains several 5′-TAAT-3′ motifs, which are binding sites for HOXD9 protein, possibly representing a target for a positive autoregulatory expression of HOX gene products. The oligonucleotide HCRII contains a 5′-TAAT-3′ motif whose mutation reduces the transcriptional effect of HOXD9 (40). *A* of Fig. 7 demonstrates that HMGY protein is able to interact with HCRII (*lane 4*). The retarded band shows a mobility higher than that due to the interaction of the GST-HOXD9 fusion protein with the HCRII oligonucleotide (*lane 2*). When both HMGY and GST-HOXD9 proteins are incubated with the oligonucleotide HCRII, only the high mo-

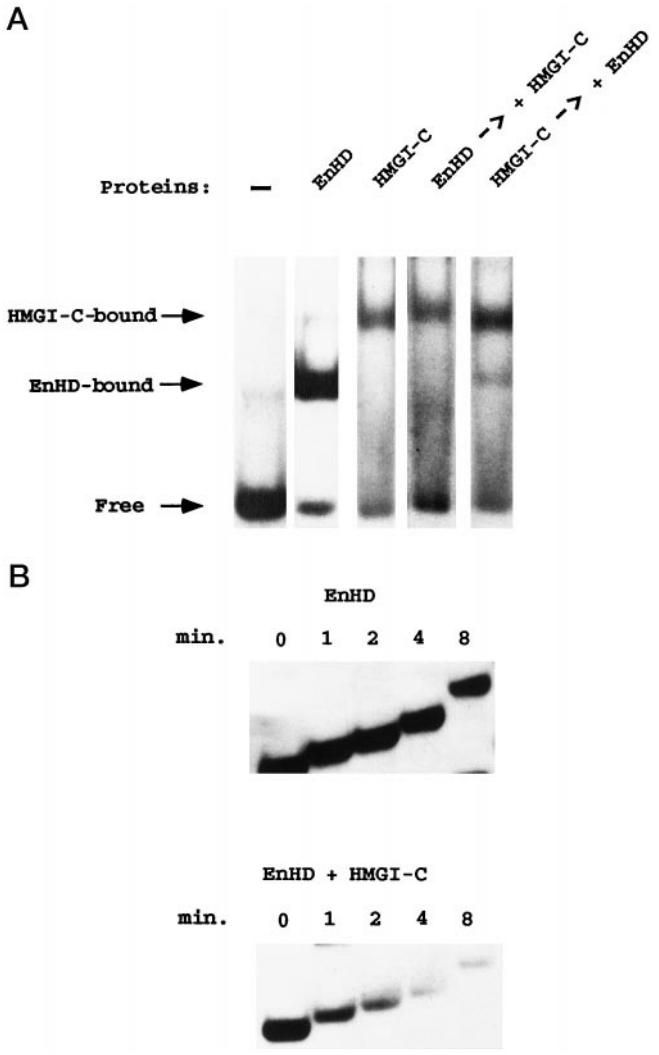

FIG. 4. **Kinetics of HMGI-C inhibitory effect on HD-CAnt1 interaction.** *A*, gel-retardation assay demonstrating that the effect of HMGI-C on HD-DNA interaction occurs adding HMGI-C to DNA either before or after the HD·DNA complex formation. *EnHD − > + HMGI-C* indicates that the addition of HMGI-C occurs after formation of EnHD·DNA complex, while *HMGI-C − > + EnHD* indicates the reverse sequence of addition. *B*, half-life of the EnHD·CAnt1 complex in absence or in presence of HMGI-C protein. EnHD and EnHD plus HMGI-C proteins were incubated for 30 min with the ^{32}P-labeled CAnt1 oligonucleotide. Subsequently, a 100-fold excess of cold CAnt1 oligonucleotide was added to the incubation mixture and then aliquots were loaded in a running native polyacrylamide gel at the indicated times.

bility band due to HMGY protein is detected (*lane 5*). To exclude that the retarded band observed in the presence of both HMGY and GST-HOXD9 is due to a trimeric complex containing both proteins, a co-incubation with a monoclonal antibody raised against the GST protein was performed. This antibody, which is able to induce a supershift when only the GST-HOXD9 protein is present (*lane 3*), is not able to produce any supershifted band in the presence of both GST-HOXD9 and HMGY proteins (*lane 6*). This result demonstrates that the retarded band, observed in the presence of both HMGY and GST-HOXD9 proteins, is due only to the former. To test if there could be a functional effect of HMGY binding to the 5′-TAAT-3′ motifs toward HOXD9-mediated activation of HCR, we then used an HCR-containing luciferase reporter: pTHCR (40), where the HCR element is fused to the herpes simplex virus thymidine kinase promoter. When pTHCR was co-transfected with different amounts of a construct expressing HOXD9 under the control of SV40 promoter, pSGD9, transcriptional activity was enhanced 4–5-fold over the basal level. Co-transfection of increasing amounts of pSGD9 in the presence of a fixed amount of pSVHMGY expressor construct, which produces HMGY protein under control of SV40 promoter, led to an inhibition, up to 50%, of HOXD9-mediated activation of HCR (Fig. 7*B*), while transfection of pSVHMGY alone did not significantly reduce the basal activity of pTHCR. The control reporter pT81luc, lacking HCR sequence, was not affected by transfection of HOXD9 and HMGY constructs, alone or in combination.

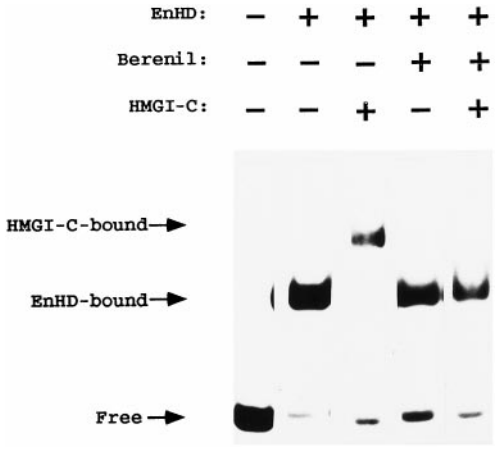

FIG. 5. **Berenil blocks the inhibitory effect of HMGI-C on EnHD-DNA interaction.** Gel-retardation assay was performed as described under "Experimental Procedures." Berenil (Sigma), HMGI-C, and EnHD were used at the concentration of 800, 0.48, and 0.25 μM, respectively.

DISCUSSION

HMGI proteins do not require a precise sequence to efficiently interact with DNA, but they do require the presence of an AT-rich stretch (14–17). This functional flexibility in terms of DNA binding could be explained by the evidence that HMGI proteins contact DNA in the minor groove, where the steric/electrostatic differences between base pairs are much less evident than those existing in the major groove (56). Due to this DNA binding flexibility, it is not surprising that HMGI proteins are able to interact with DNA sequences that are binding sites for other DNA-binding proteins. Therefore, some of the effects of HMGI proteins on gene expression may be due to binding interference with other DNA-binding proteins, through a competition for common sites.

Based on our results, the inhibitory effect of HMGI proteins on HD-DNA interaction could be described by a model in which three essential events occur: (i) HMGI proteins are able to interact with a DNA sequence already occupied by a HD-containing protein, displacing the NH_2-terminal arm of the HD from the minor groove; (ii) during the interaction, HMGI proteins induce conformational changes to the DNA; (iii) these conformational changes are not compatible with an efficient HD/DNA interaction in the major groove, therefore the whole HD-containing protein is released from the DNA. A similar model has been suggested to explain the effect of distamycin A on the Antennapedia HD·DNA complex (52). Interestingly, it has already been proposed that distamycin A and HMGI DNA binding domains have a similar planar crescent-shaped structure and can compete for the binding in the minor groove of AT-rich sequences (16). The observation that berenil, although interacting in minor groove, is not able to induce structural modifications to DNA and is not efficient in inhibiting the HD-DNA interaction, further supports our model. Crystallographic or NMR studies on HMGI proteins-DNA complexes are required to definitively prove the existence of HMGI protein-induced DNA modifications and to further test the validity of the model.

Although several experimental results indicate HMGI proteins as factors able to enhance the DNA binding of other DNA-interacting proteins (28–29), some data have been published showing an inhibitory effect of HMGI proteins on DNA binding of other factors. A clear example of this phenomenon

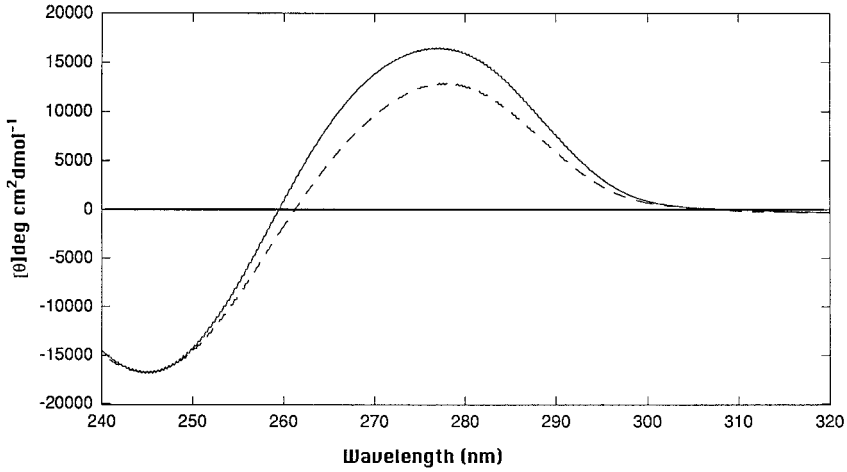

FIG. 6. **Circular dichroism spectroscopy of HMGI-C binding to DNA.** Near-UV CD spectrum of 2 μM double-stranded CAnt1 oligonucleotide ± 2 μM HMGI-C (1:1 molar ratio). The ellipticity values are given in terms of the molar concentration of oligonucleotides. The *solid line* represents the oligonucleotide alone, while the *dashed line* represents the oligonucleotide plus protein, after correction for the contribution of the protein.

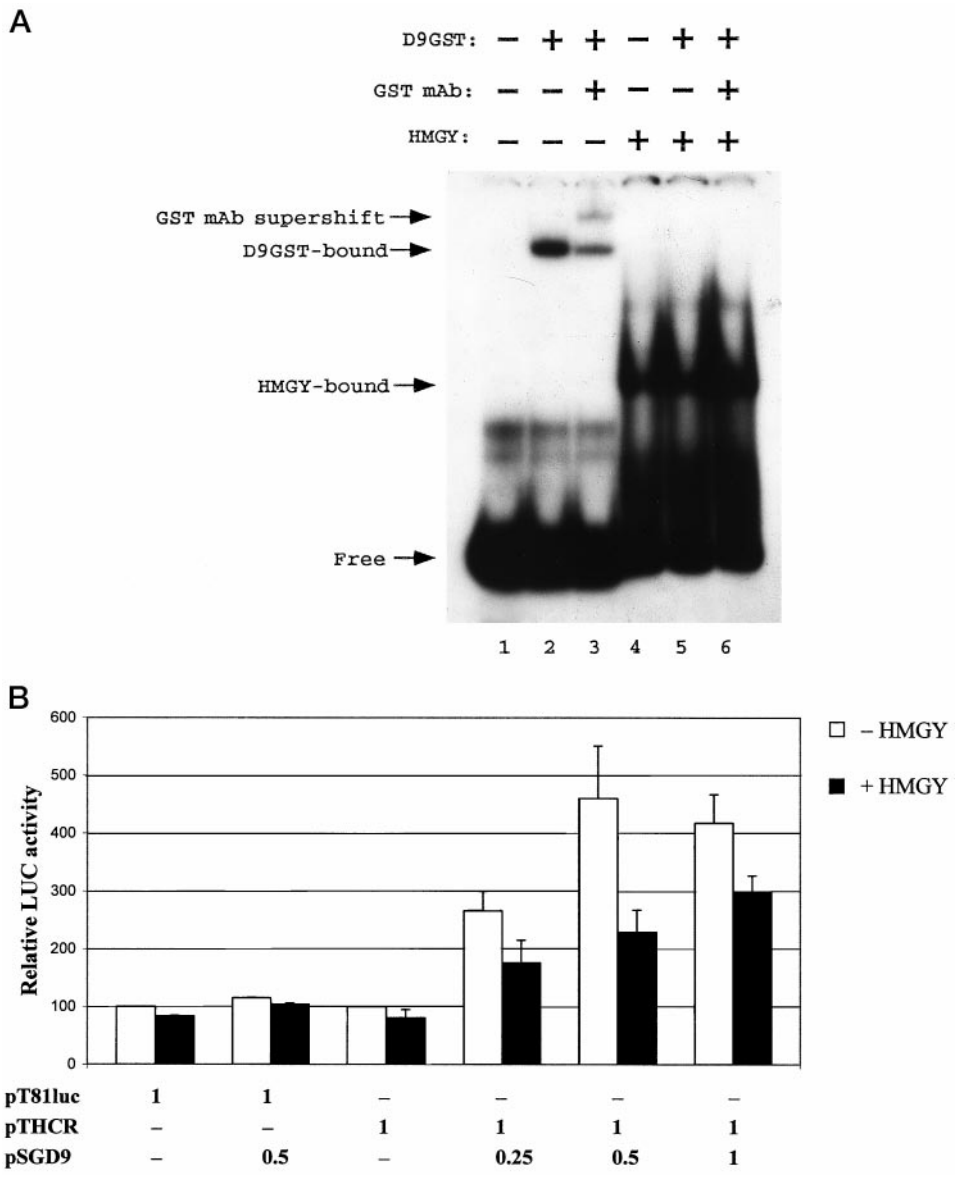

FIG. 7. **HMGY protein interferes with HOXD9 binding to DNA and inhibits its transcriptional activity.** *A*, HMGY protein binds to the oligonucleotide HCRII and abolishes the binding of GST-HOXD9 fusion protein. Gel-retardation assay was performed as described under "Experimental Procedures" using double-stranded HCRII oligonucleotide as DNA probe. HCRII was used at a final concentration of 0.02 μM, the final concentrations of HMGY and GST-HOXD9 proteins were 0.5 and 0.1 μM, respectively. *B*, NIH-3T3 cells were transiently transfected with either pT81luc or with pTHCR along with pSGD9 expression vector. The amounts indicated are expressed in micrograms. The same experiment was done in the absence (*open columns*) and in the presence (*filled columns*) of a fixed amount (5 μg) of pSVHMGY expression construct. In all experiments, total amounts of pSV and pSG vectors were kept constant by using pGDSV7 and pSG5 empty vectors, and 0.5 μg of pTK β-galactosidase plasmid were co-transfected as internal standard. Transcriptional activity is indicated as percentage of that measured for pTHCR reporter alone. *Columns* and *bars* represent the means and the S.D. values, respectively, of at least four independent experiments.

occurs in T cells at the level of the interleukin-4 promoter (57). In fact, a high HMGI(Y) concentration, typical of peripheral blood T lymphocytes, is able to displace NF-AT factors from interleukin-4 promoter, reducing both its constitutive and inducible transcriptional activity. A negative effect of HMGI(Y) on the Ig heavy chain ϵ germ line RNA promoter has been also reported (27). In this case, the inhibitory effect induced by HMGI(Y) appears to be mediated by the interaction of these proteins with a binding sequence for a STAT-like factor. A direct inhibition of Oct-1 protein DNA binding by HMGI(Y) has been also described (22). Interestingly, HMGI(Y) appears to exert distinct effects on the interaction of Oct-1 and Oct-2 proteins with the octamer sequence. In fact, while Oct-1 binding is inhibited, the presence of HMGI(Y) enhances the Oct-2/octamer sequence interaction. Such a difference may suggest that the final outcome of HMGI proteins on the transcriptional activity of factors interacting with the same or nearby sequences is due to a combination of protein/DNA and protein/protein interactions. With regard to HD-containing proteins, Zappavigna *et al.* (40) have recently reported that HMG1 is able to interact with HOX proteins, enhancing their DNA binding and transcriptional activity. This effect is mediated only via protein-protein interaction, since HMG1 alone is not able to bind to the HOX target sequence. We instead, using the same cellular system and the same vectors, show that HMGY is able to compete with HOXD9, hence mediating repression. Therefore, although opposite effects of HMG proteins on the DNA binding activity of transcriptional regulators have been reported, taken together these data point out the relevance of these architectural factors in the regulation of gene transcription.

Several studies indicate that HD-containing proteins require

regulatory events for a proper action. For instance, Castelli-Gair and Akam (58) have demonstrated that the Ultrabithorax protein elicits distinct effects in different spatial-temporal contexts of *Drosophila* embryos. The ability of HMGI proteins to negatively affect the HD binding to some DNA target sequences could be one of the mechanisms by which the same HD-containing proteins are able to induce different effects depending on the context. One intriguing possibility is that HMGI proteins provide HD-containing proteins with a sort of regulatory switch. In fact, it has been demonstrated that the binding of HMGI proteins to DNA is regulated by phosphorylation (59, 60). In particular, phosphorylation of HMGI proteins induced by $p34^{cdc2}$ kinase greatly reduces their binding to DNA. Based on our experimental results, we hypothesize that the phosphorylation/dephosphorylation of HMGI proteins controls the access of HD-containing proteins to their DNA target sequences.

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High Mobility Group I Proteins Interfere with the Homeodomains Binding to DNA

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