Distinct Organization of DNA Complexes of Various HMGI/Y Family Proteins and Their Modulation upon Mitotic Phosphorylation*

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High mobility group (HMG) proteins HMGI, HMGY, HMGI-C, and Chironomus HMGI are DNA-binding proteins thought to modulate the assembly and the function of transcriptional complexes. Each of these proteins contains three DNA-binding domains (DBD), properties of which appear to be regulated by phosphorylation. High levels of these proteins are characteristic for rapidly dividing cells in embryonic tissues and tumors. On the basis of their occurrence, specific functions for each of these proteins have been postulated. In this study we demonstrate differences in the nature of contacts of these proteins with promoter region of the interferon-β gene. We show that HMGI and HMGY interact with this DNA via three DBDs, whereas HMGI-C and Chironomus HMGI bind to this DNA using only two domains. Phosphorylation of HMGY protein by Cdc2 kinase leads to impairing of contacts between the N-terminally located DBD and a single promoter element. The perturbations in the architecture of the protein-DNA complexes involve changes in the degree of unbending of the intrinsically bent IFNβ promoter. Our results provide first insights into the molecular basis of functional specificity of proteins of the HMGI/Y family and their regulation by phosphorylation.

The family of high mobility group I/Y chromosomal (HMGI/Y)1 nonhistone proteins comprises different structurally related proteins found in evolutionary distant organisms, including bacteria, insects, plants, and mammals (1). The mammalian HMGI, HMGY (HMGI(Y)), and HMGI-C and insect cHMGI belong to the best characterized proteins of this family (1, 2). HMGI/Y play an important role in regulation of transcriptional activity of many genes (3), including those of interferon (4–6) and the receptor (7). During the last 5 years, the HMGI/Y genes have attracted a lot of interest because they are causally involved in the genesis of benign tumors. Aberrations involving the chromosomal regions 12q15 and 6p21.3 affecting the HMGI-C and HMGI(Y) gene, respectively, have been shown in a variety of benign tumors, e.g. uterine leiomyomas, endometrial polyps, pleomorphic adenomas, pulmonary chondroid hamartomas, and lipomas (8–13). At the molecular level, in case of intragenic breakpoints, different fusion transcripts have been detected as, e.g. HMGI-C-ALDH2 (14) and HMGI-C-LPP (12). In addition, a re-expression of HMGI-C and high level expression of HMGI(Y) have been observed in a number of malignant human tumors (15–20). The oncogenic potential of HMGI(Y) genes has been confirmed by functional tests including NIH3T3 assay, knock-out technique, and antisense construct transfection (21–23). Despite structural similarities between the three mammalian members of the HMGI(Y) family, it appears that each of the proteins have a specific function. In the pygmy phenotype that arises from the inactivation of the HMGI-C gene, the gene expression of the HMGI(Y) gene is not affected, thus indicating that HMGI and HMGY proteins are not able to substitute functionally for HMGI-C protein (23). Neoplastic transformation of the murine JB6 cells by a phorbol ester is accompanied by synthesis of the HMGY but not HMGI protein, suggesting that HMGI and HMGY have different functions (24). Moreover, in MCP-7 cells, posttranslational modifications appear to affect differentially the abilities of the HMGI and HMGY proteins to interact with AT-rich ligands and nucleosomes (25).

Despite continuously increasing interest in the function(s) of this group of proteins, the nature of the interaction of these proteins with DNA is still not sufficiently understood. The proteins of the HMGI/Y family are 10–11 kDa in size, are highly charged, are rich both in acidic and basic residues, are proline-rich, and contain only few residues with bulky hydrophobic side chains. This unusual amino acid composition inhibits folding of the polypeptide backbone of these proteins into any defined secondary structure. Common for HMGI/Y proteins is the presence of three putative DNA-binding domains (DBD), so called AT-hooks (26). NMR analysis of a complex of a peptide derived from HMGI(Y) bound to a short DNA fragment revealed that the centrally located RGR residues are essentially for binding and responsible for contacts of the protein with the bases and phosphate-sugar backbone (27). Alternative approaches, which used deletion and point-mutated proteins, revealed that two or three DBDs of the protein bind to DNA in a cooperative way (28–30). Application of the protein-footprinting method for mapping of protein regions interacting with DNA (31) allowed more detailed characterization of the binding of HMGI(Y) proteins to DNA (32). A combination of this method...
with other biochemical approaches has recently brought insights into the organization of the complex of the HMGI-C protein with a promoter region of the interferon-β (IFNβ) gene (33). That work revealed, that the first and the second AT-hook of the HMGI-C protein interact with PRDII and NRDI elements of the promoter (33) and that phosphorylation by Cdc2 kinase of HMGI-C perturbs organization of the protein-DNA complex. In this work we have extended our studies for other three members of the HMGI/Y family: the HMGI and HMGY as well as the insect chHMGI protein. We have found that the proteins differ in their affinities for binding to the IFNβ promoter, stabilities of the complex, and the number of protein-DNA contacts. Furthermore, we demonstrate that phosphorylation of the HMGI protein by Cdc2 kinase weakens contacts of the first AT-hook to the PRDIII-1 element of IFNβ promoter. We show that phosphorylation of the HMGI protein attenuates the unbinding activity on the IFNβ promoter. Our data support the experimental evidence that various members of the HMGI/Y family have distinct functions.

**EXPERIMENTAL PROCEDURES**

**Preparation of Bacterially Expressed Proteins**—The coding region of the human HMGI, HMGY, HMGI-C, and chHMGI proteins were cloned into the expression vector pET3a, and the proteins were overexpressed, extracted, and purified as described previously (34, 35).

**Protein Phosphorylation**—For Cdc2 kinase, 50 μg of the recombinant HMGY protein were phosphorylated at 30°C with 10 units of recombinant human Cdc2 kinase (New England Biolabs Inc.) for 5 h in the presence of 4 mM ATP in 8 μl of Cdc2 kinase buffer containing: 50 mM Tris/HCl, 10 mM MgCl₂, 1 mM ethylenedinitriloetetraacetic acid (EGTA), pH 7.5. For casein kinase 2 (CK2), 50 μg of purified mammalian HMGI protein or Cdc2-phosphorylated HMGY were phosphorylated at 37°C with 500 units of recombinant human CK2 (New England Biolabs Inc.) for 5 min in the presence of 200 μM ATP in 50 μl of CK2 buffer containing 20 mM Tris/HCl, 50 mM KCl, 10 mM MgCl₂, pH 7.5. Phosphorylation of cHMGI was extended for 2.5 h. For ³²P-end-labeling, 100–150 μCi of [γ-³²P]ATP was added to the reaction mixture. The reaction products were separated by reverse-phase high performance liquid chromatography. The identity and phosphorylation extent of the isolated proteins were confirmed by matrix-assisted laser desorption ionization/time-of-flight mass spectrometry.

**DNA and Oligonucleotides**—The synthetic linear poly(dA-dT)·poly(dA-dT) DNA was obtained from Amersham Pharmacia Biotech. The approximate average length of this DNA was 5000 bp. The 34-bp fragment of the promoter of the IFNβ gene containing the PRDIII-1, PRDII, and NRDI elements was prepared from synthetic oligonucleotides (32, 33). Four-way junction DNA (4H DNA) was prepared according to Bianchi (36). For DNA footprinting and mobility shift experiments, the oligonucleotides were ³²P-end-labeled with T4 polynucleotide kinase.

**Fig. 1** The HMGI, HMGY, HMGI-C, and chHMGI proteins interact differentially with the IFNβ promoter. A, schematic drawing showing primary structures of the mammalian HMGI, HMGY, and HMGI-C and *Chironomus* chHMGI. DBDs are indicated by boxes. Closed and open circles indicate phosphorylation sites of Cdc2 kinase and CK2, respectively. Numbers behind sequences show length in amino acid residues. B, binding of HMGI, HMGY, HMGI-C, and cHMGI to 34-bp fragment of the IFNβ promoter. <1 nM ³²P-labeled IFNβ DNA was incubated with increasing concentrations of proteins and electrophoresed for 15 min on 8% polyacrylamide gels. The gels were dried, and the radioactivity was quantified with a PhosphorImager. C, quantification of the data from B. The percentage of free DNA was plotted against ligand concentration according to Carrey (71). The lines are theoretical curves calculated from the relationship $K_d = \frac{[\text{free DNA}] \times [\text{protein}]}{[\text{complexes}]}$. The calculated $K_d$ values are shown in Table I. D, analysis of the stability of the protein-IFNβ complexes. 300 nM protein were incubated with labeled DNA, electrophoresed for various times (0.25–2 h), and analyzed as described in E. This panel is only a representative analysis for cHMGI (a), HMGY($^{[\text{P}]}$Cas2P)$^{[\text{P}]}$ (b), and HMGY (c). Identical experiments with other studied proteins are not shown. E and F, analysis of the data from D. The ratio of [bound DNA] to [bound DNA]₀ was plotted against time. 0 min indicate that the measurement starts after 15 min of electrophoresis, which was necessary for separation of free and bound DNA. The lines are theoretical curves calculated from the relationship $ln([\text{bound DNA}]_0/[\text{bound DNA}]_t) = -k_d \times t$. The calculated $k_d$ values are shown in Table I. Closed circles, HMGI; open circles, HMGY; closed triangles, HMGI-C; open triangles, chHMGI; diamonds, HMGY($^{[\text{P}]}$Cas2P)$^{[\text{P}]}$. 

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phosphates with 10% piperidine or 0.14M NaOH, respectively. Finally, eluted from the gels and cleaved at methylated purines or ethylated separated from unbound DNA by gel electrophoresis. The DNAs were trypsin in a 10-

lysine chloromethyl ketone. The cleavage with proteinases Glu-C and

were excluded from the analysis. Size markers were obtained by limited the front of the gel and those of near full-length protein, respectively,

were separated on 16.5% polyacrylamide gels using the Tricine-SDS

ties were plotted

phosphorimages of the full lane widths were scanned and the intensi-

h

were analyzed on sequencing gels. G l

absence of DNA, and

Methylation and Ethylation Interference Assays—The 5’-labeled IFNβ-DNA (30 nm) was partially digested with hydroxyl radicals in a 10-μl reaction volume in presence or absence of 30 nm HMG protein in 180 mM NaCl, 20 ng/μl bovine serum albumin, and 10 mM MOPS buffer, pH 7.2, at room temperature for 20 min as described previously (33). The reaction products were separated on 18% polyacrylamide sequencing gels containing 7 M urea/TBE. The gels were scanned and the data analyzed as described previously (33).

Mobility Shift Assay—Electrophoretic mobility shift assays were carried out as described previously (34, 35). Briefly, purified proteins were incubated with less than 1 nM of labeled DNA in 180 mM NaCl, 1 mM MgCl2, 0.01% bovine serum albumin, 8% glycerol, 10 mM Tris/HCl, pH 7.9, at 20 °C for 10 min. The DNA and DNA/protein complexes were run on 8% polyacrylamide gels at 37 °C unless otherwise indicated.

Hydroxyl Radical DNA Footprinting—10,000–15,000 cpm 5’-labeled IFNβ-DNA (30 nm) was partially digested with hydroxyl radicals in a 10-μl reaction volume in presence or absence of 30 nm HMG protein in 180 mM NaCl, 20 ng/μl bovine serum albumin, and 10 mM MOPS buffer, pH 7.2, at room temperature for 20 min as described previously (33). The reaction products were separated on 18% polyacrylamide sequencing gels containing 7 M urea/TBE. The gels were scanned and the data analyzed as described previously (33).

Hydroxyl Radical Protein Footprinting (31, 40)—10–100 pmol of the radioactively end-labeled peptide (10,000–20,000 cpm) were digested in presence or absence of DNA in a total volume of 10 μl of 180 mM NaCl and 10 mM MOPS buffer, pH 7.2, at room temperature for 30 min. The chemical digestions were started by sequential addition of 1 μl each of the following freshly prepared solutions: (i) 20 mM EDTA and 10 mM (NH4)2Fe(II)(SO4)2, (ii) 0.2 M sodium ascorbate, and (iii) 0.375% (v/v) (NH4)2Fe(II)(SO4)2, (0.375% (v/v) and 100 nM modified DNA was

m

l reaction volume in presence or absence of 30 nM HMG protein in

sera

were analyzed on sequencing gels. G l

FIG. 2. Size markers and assignment of the bands for protein footprinting of the HMGI/Y proteins. A, size markers were generated by site-specific cleavage of 32P-end-labeled HMGY with thermolysin, endoproteinases Glu-C and Arg-C, and trypsin. Hydroxyl-radical lanes show peptide patterns of the protein digested with the chemical proteinase in the absence (−) or presence (+) of DNA. A, plot of size of peptide markers versus relative mobility. Relative mobility of uncleaved HMG protein was defined as 0, and the most rapidly migrating band of hydroxyl radical cleavage as 1. Open, black, dark gray, and gray symbol correspond to peptide markers of HMGY, HMGY, HMGI-C, and HMGI, respectively.

Asp-N was carried out in the presence of 50 ng of enzyme in 25 mM sodium phosphate, pH 7.8, and 180 mM NaCl at 20 °C. Digestion of the protein in the presence of 20 ng of Arg-C was performed in 90 mM Tris/HCl containing 8.5 mM CaCl2, 5 mM dithiothreitol, and 0.5 mM EDTA, at 20 °C. Finally, the reactions were stopped by addition of 4-fold SDS sample buffer with 20 mM EDTA.

Fluorochrome-labeled DNA—The 34-bp IFNβ DNA fragment derivative with the amino-dT residues (Glen Research, Sterling, VA) incorporated into base pair 7 (bottom strand) and base pair 28 (top strand) was prepared by automated oligonucleotide synthesis. The bottom and top strands were labeled with donor and acceptor fluorochrome and were purified as described previously (42). The luminescent donor used

Architecture of the HMGI/Y-DNA Complexes

<table>
<thead>
<tr>
<th>Protein</th>
<th>“Equilibrium” dissociation constant, Kd</th>
<th>“Kinetic” dissociation constant, k_d</th>
<th>Half-lifetime of the complex</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMG</td>
<td>7.8 ± 0.4</td>
<td>18 ± 1.8</td>
<td>1.5</td>
</tr>
<tr>
<td>HMGY</td>
<td>35 ± 1.5</td>
<td>4.4 ± 1.9</td>
<td>6.3</td>
</tr>
<tr>
<td>HMGY[Pc</td>
<td>Pc]</td>
<td>107 ± 15</td>
<td>ND*</td>
</tr>
<tr>
<td>HMGY[P</td>
<td>Pc</td>
<td>Pc]</td>
<td>330 ± 15</td>
</tr>
<tr>
<td>HMGY-C</td>
<td>60 ± 3.0</td>
<td>7.1 ± 0.2</td>
<td>3.9</td>
</tr>
<tr>
<td>chHMGI</td>
<td>17 ± 1.1</td>
<td>16 ± 2.0</td>
<td>1.7</td>
</tr>
</tbody>
</table>

* ND, not determined.
an excitation source (43). Donor emission was observed at 617 nm, whereas acceptor emission was observed at 670 nm. The details of the luminescence resonance energy transfer (LRET) measurements and the advantages of using europium chelates for energy transfer measurements have been discussed previously (43, 46–50). Only sensitized acceptor decays were analyzed since these decays contain information pertaining to energy transfer in the absence of any contamination with the signal of the donor not engaged in energy transfer (42–45). The sensitized acceptor decay curves were analyzed according to Equation 1.

\[
I = S_0 \exp(-t/t_i)
\]

(Eq. 1)

\[
E = 1 - t_a/t_s
\]

(Eq. 2)

The distances between donor and acceptor were calculated according to Förster theory (52).

\[
R^6 = R_0^6/(1 - E/E)
\]

(Eq. 3)

\( R \) is a distance between a donor and an acceptor, and \( R_0 \) is a distance at which the energy transfer is 0.5. The \( R_0 \) was calculated assuming a completely randomized orientations of donor and acceptor. This assumption is particularly valid in the case of long-lived europium chelate donors (47–49) and practically eliminates the uncertainty of distance measurement due to unknown mobility of fluorochromes.

RESULTS

High Mobility Group Proteins HMGI, HMGY, HMGI-C, and chHMGI Interact Distinctly with Interferon \( \beta \) Promoter—Studies in which properties of the all three mammalian HMGI/Y proteins have been compared are rare. Maher and Nathans (29) were the first who simultaneously analyzed binding properties of HMGI, HMGY and HMGI-C. However, the authors focused their work on the role of the length and the spacing of AT-tracts of HMGI, HMGY and HMGI-C, respectively (Fig. 1, B and C; Table I). The strong binding of chHMGI protein to this promoter may suggest that function(s) of cHMGI are similar to that of HMGI and HMGY in mammals (Fig. 1, B and C; Table I).

During migration through the gel, the protein-DNA complex is under nonequilibrium conditions and dissociates continuously (Fig. 1D). Analysis of the process allows obtaining information on the stability of the complex, e.g. its susceptibility to dissociation in response to changing physiological conditions. Quantification of the amounts of the labeled DNA in the complex after various times of electrophoresis (Fig. 1D) allowed determination of kinetic dissociation constant \( (k_d) \) for each protein (Fig. 1, E and F; Table I). The HMGY protein has the

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2 T. Heyduk, manuscript in preparation.
In the absence or presence of protein, the reaction products were separated on a sequencing gel and scanned with a PhosphorImager, and bands were quantified with ImageQuant software. Each bar shows relative cutting frequency at a single base. 100% cutting frequency corresponds to digestion of the DNA fragment in the absence of protein, so that lower values mean protection upon protein binding. The concentrations of the DNA and proteins were 30 nM. The top and bottom strand sequences of the IFN\(\beta\) promoter are shown between the panels.

Proteins HMGI, HMGY, HMGI-C, and cHMGI Bind Differentially to IFNb Promoter—Hydroxyl radical protein footprinting experiments with HMGI, HMGY, HMGI-C, and cHMGI proteins were performed to identify regions of the proteins involved in contacts with the fragment of the promoter of the IFN\(\beta\) gene. Phosphorylation with CK2 kinase of the sites located in the C-terminal region of each protein was used for the end-labeling of the proteins (Fig. 1A). This phosphorylation reflects a constitutive modification found in native forms of these proteins (53–55). The free and DNA-bound proteins were digested with hydroxyl radicals, and the digestion products were separated by electrophoresis. The gels were calibrated using arrays of peptides generated by limited digestion of the labeled proteins by different proteinases. As an example, digestion patterns of the HMGY protein are shown in Fig. 2 (A–D). Relative mobilities of the digestion products were transformed by nonlinear regression into residue positions within the protein, which allowed the alignment of the hydroxyl radical products (Fig. 2B). Since the changes in the cleavage pattern are usually small (Fig. 2A), the radioactivity in the lanes was scanned, and the intensities were transformed into difference plots (Fig. 3, A–D). This analysis revealed that each of the proteins has an individual protection/exposition pattern (Fig. 3, A–D), indicating that each protein-DNA complex has a specific topology and a characteristic extent of contacts between the protein and the promoter fragment. The differences between HMGI and HMGY proteins are essentially moderate and reflect high similarity between these proteins. In the presence of DNA, the HMGI protein was protected mainly in the region of the DBD2 and to lower extent within the other two AT-hooks (Fig. 3B). In contrast, all three AT-hooks in the HMGI-DNA complex were similarly protected (Fig. 3B). In agreement with our previous data (33), footprinting of HMGI-C revealed that only DBD1 and DBD2 of this protein are involved in contacts with the DNA fragment and that in the complex a large segment between the DBD2 and DBD3 is strongly exposed to the solvent (Fig. 3C). Binding of cHMGI protein involved the second and the third DBD of the protein (Fig. 3D).

To extend the insights in the organization of the individual HMGI/Y proteins-DNA complexes DNA-footprinting (Fig. 4) and interference assays (Fig. 5) using methylated and ethylated DNAs have been performed. Our previous studies revealed that HMGI-C protein binds to the PRDII and NRDI elements of this DNA. In contrast, binding of the HMGI protein resulted in the protection of three regions of the DNA, including additionally the PRDIII-1 element (Fig. 4A). The presence of HMGI protein, protection of both DNA strands in the PRDII and NRDI region was observed (Fig. 4), whereas the protection in the PRDIII-1 element was restricted to the bottom strand (Fig. 4B). Essentially the same results were obtained using up to 10 times higher protein concentrations (data not shown). Ethylation interference assay showed that modification of majority of phosphate groups in the DNA fragment interferes with binding. However, strongest interference in binding of both proteins was observed in region of the PRDII element (Fig. 5A). Modifications within the NRDI-element affect the binding of HMGY somewhat more as compared with...
HMGI (Fig. 5A). A moderate extent of interference within the PRDIII-1 was found for both proteins (Fig. 5A). Similar results were obtained analyzing the binding interferences on the bottom strand (data not shown). Methylation of purines interfered to higher extent with binding of DNA fragments modified at adenines located within PRDII and NRDI (Fig. 5B). No interference in binding was observed for fragments methylated at purines within the PRDIII-1 region (Fig. 5B). These data suggest that interaction of HMGY and HMGI in the PRDIII-1 region is probably mostly nonspecific by interaction with the phosphate backbone and therefore detectable by footprinting and ethylation interference techniques but is not affected by individual modification of the bases.

In the DNA footprinting experiments with HMGI and HMGY, the distances between the minima of the cutting frequency are 10–11 and 7–8 bases between NRDI - PRDII and PRDII-PRDIII-1, respectively (Fig. 4). A similar distance of 7–8 bases between the minima within PRDII and PRDIII-1 was also observed in ethylation interference experiment (Fig. 5A). Thus, the centers of binding to NRDI-PRDII and PRDII-PRDIII-1 appear to be separated by one and approximately three-quarters helical turns, respectively. This suggest that the center of protein binding to the PRDIII-1 is rotated by about 90° in respect to the binding at PRDII and NRDI.

In the presence of Chironomus cHMGI protein the patterns of footprinting were distinct from those obtained for the human proteins (Fig. 4). Strong protection within PRDII element was observed on both strands. Moreover, wide regions of the top and bottom strands, respectively, comprising NRDI and PRDIII-1, were protected. Essentially the same protection patterns were found and higher up to 10:1 protein to DNA ratios (not shown).

Phosphorylation Affects Contacts of HMGY with DNA—Previous studies have shown that mammalian and insect HMGI/Y proteins are in vivo phosphorylated at several positions by CK2 (53–55) and Cdc2 kinase (56–58). In some reports, a general phosphorylation-dependent attenuation of binding affinity of the proteins has been demonstrated (56–60); however, the mechanism of these changes was not studied. Recently, we have shown that phosphorylation of the HMGI-C protein by Cdc2 kinase “derails” DBD2 of this protein from the minor groove (33). In this work we have observed differences in the organization of complexes of human HMGI-C and HMGI(Y) with IFNβ promoter. Therefore, we decided to study more detailed how phosphorylation by the Cdc2 kinase affects binding of the HMGY protein to DNA.

An approximately 3-fold weakening of binding of HMGY to IFNβ was found upon phosphorylation of the protein at Ser-87, Ser-90, and Ser-91 by CK2 (Table I). Further phosphorylation by Cdc2 kinase additionally weakened the interaction of the protein with this DNA (Table I). Analysis of the binding of the protein to 4H DNA, which is a well characterized ligand of the HMGY protein (28, 61, 62) revealed at least 10-fold reduction of the affinity of the CK2 phosphorylated protein (Fig. 6, A and B). Consecutive phosphorylation by Cdc2 kinase further reduced the strength of the binding (Fig. 6C). Interestingly, phosphorylation by Cdc2 kinase had influence on the mobility of DNA.

**Fig. 6.** Effect of phosphorylation of HMGY on binding to 4H DNA. <1 nM 32P-labeled 4H DNA was incubated with increasing concentrations of nonphosphorylated and phosphoforms of HMGY, and electrophoresed on 8% polyacrylamide gels at 20 °C. The gels were dried and autoradiographed. Differences in the mobility of 4H DNA bound to HMGY[P<sup>CK2</sup>] and HMGY[P<sup>CK2</sup>P<sup>Cdc2</sup>] are indicated by arrows to the left of panel C. The protein concentrations were: a, 0.3; b, 1; c, 3; d, 10; e, 30; f, 100 nM.

**Fig. 7.** Hydroxyl radical protein footprinting of poly(dA-dT)poly(dA-dT) (A and B), IFNβ fragment (A and C), and 4H DNA (A and D) on the end-labeled HMGY protein. A, representative electrophoretic patterns of hydroxyl radical digestions of the HMGY[P<sup>CK2</sup>] and HMGY[P<sup>CK2</sup>P<sup>Cdc2</sup>] proteins in the absence (−) or presence (+) of poly(dA-dT)poly(dA-dT) (64 bp/molecule HMGY), IFNβ fragment (2:1 DNA:protein), or 4H DNA (2:1 DNA:protein). B–D, difference plots showing averaged data from four independent experiments. Results of HMGY[P<sup>CK2</sup>] and HMGY[P<sup>CK2</sup>P<sup>Cdc2</sup>] are shown in black and gray lines, respectively. Schematic primary structure of the HMGY protein with DBD (boxes) are shown in the lower part of the panels (B–D).

by Cdc2 kinase.
proteins were 30 nM.

To map changes resulting from Cdc2-phosphorylation of HMGY, the protein was subjected to hydroxyl radical footprinting in the presence of IFNβ and 4H DNA. We also analyzed binding of the protein to poly(dA-dT) because earlier work showed that HMGI(Y) proteins bind strongly to this type of DNA (63). Protein footprinting of HMGY in the presence of poly(dA-dT) and IFNβ promoter fragment revealed that three regions of the HMGY protein carrying AT-hooks were protected from hydroxyl radical digestion (Fig. 7, A–C). In both complexes, phosphorylation of the protein by Cdc2 kinase led to weakening of the DBD1 binding and an substantial increase of protection in the region containing DBD3 (Fig. 7, A–C). This observation correlates well with a previous demonstration that the HMG1 protein binds to this 4H DNA at two positions on opposite arms of the junction (61, 62). Phosphorylation by Cdc2 kinase impaired the binding of DBD2, whereas contacts of the DBD3 appeared somehow increased (Fig. 6C). Since the reduction of the mobility of the complex cannot be directly attributed to the decrease of the charge of the protein, which should increase the mobility of the complex, it appears that the change in the mobility reflects alterations in the organization of the complex. Either changes in the complex stoichiometry or changes in conformation of 4H DNA are possible and require further analysis.

To measure the unbending potential of HMGY and its phosphorylated forms, we have constructed the 34-bp IFNβ fragment with Eu³⁺-DPTA-AMCA donor and CY5 acceptor molecules separated by 2 helical turns of DNA and located in major grooves on the opposite face of DNA with respect to where the proteins bind (Fig. 9A). The distance between fluorophores is 20 bp; thus, in B-DNA, the distance between them would be $R_{d} = 6.8$ nm. Assuming that the total bend between the position of the fluorophores is $20^\circ$, the changes in the bend angle upon protein binding can be calculated using the equation shown in C. $R_{d}/2C$, the distance calculated from energy transfer measurements, $D$, an example of sensitized acceptor decay of donor- and acceptor-labeled 34-bp DNA fragment. The curve shown is for the DNA in the absence of any protein. The solid line is a nonlinear regression fit to two-exponential decay model. Inset, comparison of decay-curves for LRET of the DNA in the absence (solid line) and presence (broken line) of HMGY. E, the quality of the fit is demonstrated by random distribution of residuals and by a value of $\chi^2$, close to 1. The quality of the fits observed with every protein studied was comparable to an example shown in this figure.

**Fig. 8.** Hydroxyl radical DNA footprints of the HMGY[PCK2] and HMGY[PCK2+PCK2] proteins on the IFNβ fragment. End-labeled DNA (top strand) was digested with hydroxyl radicals in the absence or presence of HMGY[PCK2] (black bar) or HMGY[PCK2+PCK2] (gray bar). The reaction products were separated on a sequencing gel and scanned with a PhosphorImager, and bands were quantified with ImageQuant software. Each bar shows relative cutting frequency at a single base. 100% cutting frequency corresponds to digestion of the DNA fragment in the absence of protein, so that lower values mean protection upon HMGY binding. The concentrations of the DNA and proteins were 30 nM.

**Fig. 9.** Luminescence resonance energy transfer measurements. A–C, rationale of the LRET-based assay for measurement of unbending of IFNβ-promoter. A, the donor and acceptor moieties are located in the major groove of the PRDIII-1 and NRDI-1. The distance between fluorophores is 20 bp; thus, in B-DNA, the distance between them would be $R_{d} = 6.8$ nm. Assuming that the total bend between the position of the fluorophores is $20^\circ$, the changes in the bend angle upon protein binding can be calculated using the equation shown in C. $R_{d}/2C$, the distance calculated from energy transfer measurements. D, an example of sensitized acceptor decay of donor- and acceptor-labeled 34-bp DNA fragment. The curve shown is for the DNA in the absence of any protein. The solid line is a nonlinear regression fit to two-exponential decay model. Inset, comparison of decay-curves for LRET of the DNA in the absence (solid line) and presence (broken line) of HMGY. E, the quality of the fit is demonstrated by random distribution of residuals and by a value of $\chi^2$, close to 1. The quality of the fits observed with every protein studied was comparable to an example shown in this figure.
and were larger than the error of the measurement. Table II summarizes the results of distance measurements. Assuming a model shown in Fig. 9 (A–C) and using a prediction of the curvature of the DNA (65) between the donor and acceptor yielded a value of a mean value of −10° per helical turn (33). Binding of HMGY unbends the DNA by −7°, whereas consecutive phosphorylation of the protein by CK2 and Cdc2 kinase led to partial restoration of the bend by −2° and −3.5°, respectively (Table II). Interestingly, substantially weaker effect of HMGI-C on the DNA curvature was observed (unbending by −3°). This probably reflects that IFN-γ gene is not a primary target for HMGI-C. Binding a HMGI/Y-related protein, HMGIY-L1 (66), in which two of the three AT-hooks are mutated and do not bind tightly to this DNA (67), has no effect on the bend angle of the DNA.

**DISCUSSION**

The presence of three AT-hooks in each of the human and murine HMGI/Y proteins, as well as similarities in the regions adjacent to the respective DBDs and the presence of the acidic tails, have usually been a reason for a consideration that members of this protein family have similar properties. One of the significant findings emerging from this study is the demonstration of differences in DNA binding of individual proteins of the HMGI/Y family. The proteins are distinct in DNA binding affinities and thermodynamic stabilities of the complexes. Moreover, the use of only two DBDs by HMGI-C and cHMGI or three DBDs by HMGI and HMGY (Fig. 10) is probably important for a creation of an unique conformation of the bound DNA, which may facilitate recruitment of other proteins involved in assembly of multiprotein promoter/enhancer complexes as it has been well documented for the IFN-β-enhancerosome (4, 6). This study shows that HMGI binds to IFN-β promoter with the highest affinity, whereas the other two human proteins, HMGY and HMGI-C, bind less tightly. This appears as a logical consequence because usually HMGI is more abundant than HMGY and the occurrence of HMGI-C is restricted to early development or neoplastically transformed cells. An inverse situation was found for a promoter that is regulated by HMGI-C. The HMGI-C protein exhibited the highest binding strength to this promoter, whereas binding of HMGI was less tight. Thus, it seems that each of the HMGI/Y proteins plays a specific role in gene regulation by activation/inactivation of a specific set of genes. The findings that HMGY cannot substitute HMGI-C in the pygmy phenotype (23) and that HMGI and HMGY translation and/or stability is differentially regulated (24) are in agreement with this postulate.

As DBDs of the human HMGI, HMGY and HMGI-C protein are nearly identical, the differences in the binding of these proteins to particular DNAs appear to result from the other parts of these proteins. In this respect, both the length of the polypeptide chain between the DBDs and its primary structure seem to be important. The differences in the binding properties between HMGI and HMGY must be attributed to the presence of additional 11 apolar residues between the first and the second AT-hook in the HMGI protein. The HMGI-C protein is rich in glutamine (12%, versus 5% in HMGI), a residue that can form two hydrogen bonds with adenine and guanine in the major and minor groove, respectively (68). Moreover, the absence or presence of leucine and isoleucine residues in HMGI(Y) proteins versus absence of these residues in HMGI-C, may also influence the nature of how these proteins interact with specific DNA.

The properties and specific functions of majority of HMG proteins are probably regulated by phosphorylation (55). In respect to HMGI/Y family, CK2 and Cdc2 kinase appear to have a pivotal position. Whereas CK2 phosphorylation plays obviously a constitutive role in protein conformation and sta-
The phosphorylation of the HMGY protein at residues adjacent to DBD2 and DBD3 by Cdk2 kinase affects mainly binding of the DBD1 (Fig. 10). This finding emphasizes the cooperative nature of DNA binding of the AT-hooks in proteins of the HMGI(Y) family. Moreover, since this binding domain is located distantly from the phosphorylation sites (in the primary structure scale), our finding stresses the advantage of studying protein-DNA interactions of this type of proteins using entire and native molecules over using peptides derived from the sequence. In HMGI-C the Cdk2 phosphorylation sites flank the DBD2, and the phosphorylation affects mainly contacts of DBD2 with the minor groove of the PRDII element (33).

The native binding sites of the HMGI(Y) proteins in the promoter/enhancer regions of many genes targets have been described (1, 2). In contrast, target genes for HMGI-C are still not reported. A number of genes that are up- or down-regulated by a HMGI-C gene expression have been found recently using the micro-array technique.4 By means of the methods employed in this study and using appropriate regions of these putative genes, it could be possible to analyze the organization of a native HMGI-C complex. Furthermore, studies in which the structural changes upon binding of mutants of HMGI-C protein that are found in many tumors could contribute to understanding molecular events important for the role of this group of HMGI proteins in neoplasia.

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REFERENCES


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