Architecture of High Mobility Group Protein I-C·DNA Complex and Its Perturbation upon Phosphorylation by Cdc2 Kinase*

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The high mobility group I-C (HMGI-C) protein is an abundant component of rapidly proliferating undifferentiated cells. High level expression of this protein is characteristic for early embryonic tissue and diverse tumors. HMGI-C can function as an architectural factor enhancing the activity of transcription factor NF-κB on the β-interferon promoter. The protein has three minor groove DNA-binding domains (AT-hooks). Here, we describe the complex of HMGI-C with a fragment of the β-interferon promoter. We show that the protein binds to NRDI and PRDII elements of the promoter with its first and second AT-hook, respectively. Phosphorylation by Cdc2 kinase leads to a partial derailing of the AT-hooks from the minor groove, affecting mainly the second binding domain. In contrast, binding to long AT stretches of DNA involves contacts with all three AT-hooks and is marginally sensitive to phosphorylation. Our data stress the importance of conformation of the DNA binding site and protein phosphorylation for its function.

High mobility group proteins are abundant components of chromatin, which modulate DNA conformation and facilitate assembly of higher order structures (for a review, see Refs. 1 and 2). A subgroup of these proteins, the HMGI(Y)[1] family, comprises diverse proteins containing short DNA-binding domains (AT-hooks; Ref. 3) and acidic C-terminal regions. They act as regulatory factors affecting indirectly the expression of genes (for a review see Ref. 4). High levels of the proteins were found in transcriptionally active chromatin (5) and in constitutive heterochromatin of metaphase chromosomes (6). In mammalian cells three proteins of this type were detected, the HMGI, HMGY, and HMGI-C (7, 8). They are abundantly expressed in embryonic, rapidly proliferating, and tumor cells. Rearrangements or impairing of the *HMGI-C* gene were found in a number of tumors of mesenchymal origin (9, 10) and lead to the *pygmy* phenotype (11), respectively. These observations emphasize involvement of this protein in cell growth and development.

However, although different binding sites of HMGI(Y) proteins within gene enhancers and promoters have been described, the organization of this protein-DNA complex is poorly understood. The proteins have three putative AT-hooks that interact with the minor groove of DNA (12). Two of them are necessary for strong binding to DNA (13–15), and the involvement of particular AT-hooks depends on the DNA conformation (16). NMR studies of HMGI revealed that, in the protein-DNA complex, the central portion of the AT-hook, the tripeptide RGR, penetrates the minor groove of the AT-rich stretches, whereas residues on both sides of the peptide establish extensive contacts with the sugar-phosphate backbone (17). On the basis of the DNA-binding strength and extent of the contacts to the backbone, these DNA binding domains (DBD) were classified as type I DNA binding domains (I-DBD) and type II DBD with high and low binding affinity, respectively (17).

The proteins of the HMGI(Y) family are phosphoproteins. They are phosphorylated by protein kinase CK2 (18, 19) and, in a cell-cycle- and differentiation-dependent manner, by Cdc2 kinase (20–22), mitogen-activated protein kinase, and protein kinase C (22). *In vivo* the entire population of the HMGI-C protein appears to be phosphorylated (23). This protein possesses four putative phosphorylation sites for CK2 located within its C-terminal acidic tail and two phosphorylation sites for Cdc2 kinase flanking both sides of the central AT-hook (Fig. 1*A*) (24). The modification within the C-terminal region of the protein by CK2 has a constitutive character, and it appears to be important for proper conformation and function since phosphorylation by this kinase reduces the DNA-binding affinity of the HMGI protein (25) and increases resistance of insect HMG proteins to proteinases (26). Phosphorylation by Cdc2 reduces binding strength of the mammalian and insect HMGI proteins to DNA (20–22).

In this work we describe the organization of the complex of the murine HMGI-C protein with a 34-bp DNA fragment of the β-interferon gene promoter. We show that the N-terminally and the centrally located AT-hooks of the protein interact with the NRDI and PRDII promoter elements, respectively. The third AT-hook and the PRDIII-1 element of the 34-bp fragment do not contribute to protein-DNA interaction. Phosphorylation of the protein by Cdc2 kinase impairs a number of contacts of the central AT-hook with the PRDII element. In contrast binding of the HMGI-C protein to poly(dA-dT)·poly(dA-dT) involves all three AT-hooks and is only weakly affected by phosphorylation. The data presented allow insights in the nature of the HMGI-C protein binding to various DNAs and demonstrate how this interaction is modulated by Cdc2 kinase.

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[1] The abbreviations used are: HMGI, high mobility group I; HMGY, high mobility group Y; HMGI-C, high mobility group I-C; DBD, DNA binding domain; MOPS, 4-morpholinepropanesulfonic acid; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; IFN, interferon; PRDII and PRDIII, positive regulatory domain II and III, respectively; NRDI, negative regulatory domain.

EXPERIMENTAL PROCEDURES

Preparation of Bacterially Expressed HMGI-C and Protein Determination—The plasmid coding for murine HMGI-C has already been

described (27). The protein was purified by reverse-phase high performance liquid chromatography on a Bio-Rad RP304 column as described elsewhere (23). The consistency between purified recombinant HMGI-C protein and calculated molecular mass from sequence was checked by mass spectrometry (Perkin-Elmer API 1 spectrometer). The concentration of HMGI-C was obtained by measuring the tryptophan absorbance at 280 nm using an absorption coefficient for tryptophan of 5500 M^{-1} cm^{-1}.

Protein Phosphorylation by Cdc2 Kinase—Fifty μg of the recombinant HMGI-C protein were phosphorylated at 30 °C with 10 units of recombinant human Cdc2-kinase (New England Biolabs) for 5 h in the presence of 4 mM ATP in 8 μl of Cdc2 kinase buffer containing: 50 mM Tris/HCl, 10 mM MgCl$_2$, 1 mM dithiothreitol, 1 mM EGTA, pH 7.5.

Protein Phosphorylation within Its C-terminal Region by Casein Kinase 2—Twenty-five μg of HMGI-C or Cdc2-phosphorylated HMGI-C proteins 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 MgCl$_2$, pH 7.5. For ^{32}P end-labeling, 100–150 μCi of [γ-^{32}P]ATP was added to the reaction mixture.

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 IFN-β gene containing the PRDIII-1, PRDII, and NRDI elements and a DNA with the same base composition and length as the IFN-β fragment, but lacking the AT-tracts (P-DNA) were prepared from synthetic oligonucleotides (Fig. 1*C*). For DNA footprinting the oligonucleotides were ^{32}P-end-labeled with T4 polynucleotide kinase. The DNAs were purified by TBE polyacrylamide gel electrophoresis.

Hydroxyl-radical Protein Footprinting—Ten pmol of the radioactively end-labeled protein (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 (NH$_2$)$_2$ Fe(II)(SO$_4$)$_2$, (ii) 0.2 M sodium ascorbate, and (iii) 0.375% (v/v)H$_2$O$_2$. Reactions were stopped after 30 min by addition of 3.3 μl of 4-fold SDS sample buffer (4% SDS, 16% glycerol, 25 mM Tris/HCl, pH 6.8, 6% β-mercaptoethanol, and 0.01% bromphenol blue). The reaction products were separated on 16.5% polyacrylamide gels using the Tricine-SDS buffer system (28). The gels were dried and scanned using a PhosphorImager.

Size Markers and Assignment of the Hydroxyl-radical Cleavage Sites—Size markers were obtained by limited digestions of 10 pmol of end-labeled HMGI-C protein by trypsin, thermolysin, proteinase Arg-C, or proteinase Glu-C (V8) in 10 μl of reaction volume (all enzymes were obtained from Roche Molecular Biochemicals). Cleavage in the presence of 10 ng of trypsin or thermolysin was carried out in 180 mM NaCl, 20 mM Tris/HCl, pH 7.5 at 0 °C or 20 °C, respectively. The reactions with trypsin were stopped by addition of 1 μl of 0.14 mM N^α-p-tosyl-L-lysine chloromethyl ketone. The cleavage with proteinase Glu-C (V8) 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 CaCl$_2$ 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. The end-labeled peptide 82–107 was prepared by cleavage of the protein by iodosobenzoic acid (29).

Data Analysis of Protein Footprinting—The phosphorimages were essentially analyzed according to Heyduk *et al.* (30) and Baichoo and Heyduk (31) as described previously by Frank *et al.* (16). Due to ambiguity of the assignment and poor resolution of the peptides at the front of the gel and those of near full-length protein, respectively, regions 1–9 and 87–107 were excluded from the analysis.

Hydroxyl-radical DNA Footprinting—10,000–15,000 cpm 5′-labeled IFN-β-DNA (30 nM) was partially digested in 10 μl of reaction volume in presence or absence of 60 nM HMGI-C[P^{CK2}] or HMGI-C[P^{Cdc2},P^{CK2}] in 180 mM NaCl, 20 ng/μl bovine serum albumin, and 10 mM MOPS buffer, pH 7.2 at room temperature for 20 min. The chemical digestions were started as described under "Hydroxyl-radical Protein Footprinting." The reactions were stopped by addition of 150 μl of 0.3 M sodium acetate, pH 7.0, 0.1 mM EDTA, 100 μg/ml tRNA, and 50 mM thiourea and subsequent ethanol precipitation. Reaction products were solubilized in formamide loading buffer (80% formamide, 10 mM NaOH, and 1 mM EDTA) and were separated on 18% polyacrylamide sequencing gels containing 7 M urea/TBE. The gels were scanned with a PhosphorImager (Molecular Dynamics), and lanes were aligned with ALIGN

software. Bands were assigned by Maxam-Gilbert G+A standard, and their intensities were integrated after subtracting the background. Gel loading and cleavage efficiencies were normalized and relative cutting frequency of digestion without protein was set to 100% at every single base.

Mobility Shift Assay—Electrophoretic mobility shift assays were carried out as described previously (32, 33). Briefly, purified proteins were incubated with less than 1 nM of labeled DNA in 180 mM NaCl, 1 mM MgCl$_2$, 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.

Methylation Interference Assay—The 5′-labeled IFN-β-DNA was methylated with dimethyl sulfate (34). 500 nM modified DNA was incubated with 1 μM HMGI-C[P^{CK2}] or HMGI-C[P^{Cdc2},P^{CK2}], and the protein-DNA complexes were separated from unbound DNA by gel electrophoresis. The DNAs out of the complexes were eluted from the gels and cleaved at methylated purines with piperidine. Finally, equal amounts of radioactivity (~5000 cpm) of the cleavage products were analyzed on sequencing gels. G+A standard was generated according to Maxam and Gilbert (35).

Site-specific Protein-DNA Photocross-linking (36)—10 μM HMGI-C[P^{CK2}] or HMGI-C[P^{Cdc2},P^{CK2}] were derivatized at Cys-40 with 300 μM 4-azidophenacyl bromide in 20 mM Tris/HCl, pH 7.9, 0.2 M NaCl, 0.1 mM EDTA, 5% glycerol, and 1% dimethyl formamide in 100 μl of reaction volume for 8 h at room temperature. The reaction products were purified immediately by reverse phase-high performance liquid chromatography as described above. 15–30 nM 5′-labeled IFN-β DNA were incubated with 200 nM azidophenacyl bromide HMGI-C-derivative in 50 μl of 180 mM NaCl, 20 ng/μl bovine serum albumin, and 10 mM MOPS buffer, pH 7.2 for 10 min. The mixture was irradiated with UV light on a bench transilluminator (Herolab UVT 2020) for 15 s in a polystyrene cuvette (λ > 290 nm). Resulting covalent DNA·HMGI-C complexes were incubated with 0.1% SDS and were phenol-chloroform-extracted (4:1). Phenol phases containing the complexes were ethanol-precipitated and dried. For strand scission at the cross-linking positions, the pellets were diluted in 100 μl of NH$_4$CH$_3$COO, 0.1 mM EDTA, 2% SDS at 90 °C for 15 min; NaOH was added to a final concentration of 0.1 M; and reaction mixtures were incubated for 30 min at 90 °C. Reactions were stopped by addition of 100 μl of 20 mM Tris/HCl, pH 7.9, and 1 μl of 12 M HCl; products were ethanol-precipitated and analyzed on a sequencing gel as described above.

RESULTS

Design and Properties of the DNA Fragments Used—In some cases local intrinsic bending of DNA is important to target transcriptional regulators and to assemble higher order structures. Binding of the HMGI(Y) proteins to the intrinsically prebent elements within the promoter of the β-interferon gene, which facilitate the assembly of the enhanceosome (37), is a well characterized example for such processes. Similarly to HMGI(Y) proteins (38), the HMGI-C protein enhances transcriptional activation of β-interferon gene mediated by NF-κB (39). It binds to the promoter fragment containing the PRDII and NRDI elements (14).

To characterize the interaction of the HMGI-C protein (Fig. 1*A*) with DNA, we used a 34-bp promoter fragment from the β-interferon gene containing PRDIII-1, PRDII, and NRDI elements (IFN-β fragment; Fig. 1*C*). Furthermore, we analyzed binding of the protein to a DNA fragment of the same length and base pair composition as the IFN-β fragment, but with another sequence (P-DNA; Fig. 1*C*). The IFN-β fragment contains three AT stretches located at one face of the DNA. Circular permutation analysis revealed a 20° curvature of the PRDII element (40). A curvature prediction according to sequence-dependent anisotropic bendability model using the consensus method (41) indicates that curved conformation extends over the majority of the base pair steps in the IFN-β fragment (Fig. 1*D*, *solid line*). In contrast the prediction for the P-DNA indicated that this fragment is essentially straight (Fig. 1*D*, *dashed line*).

Phosphorylation of HMGI-C by CK2 and Cdc2 Kinase—To obtain end-labeled HMGI-C for protein footprinting experiments, the bacterially expressed protein was phosphorylated *in*
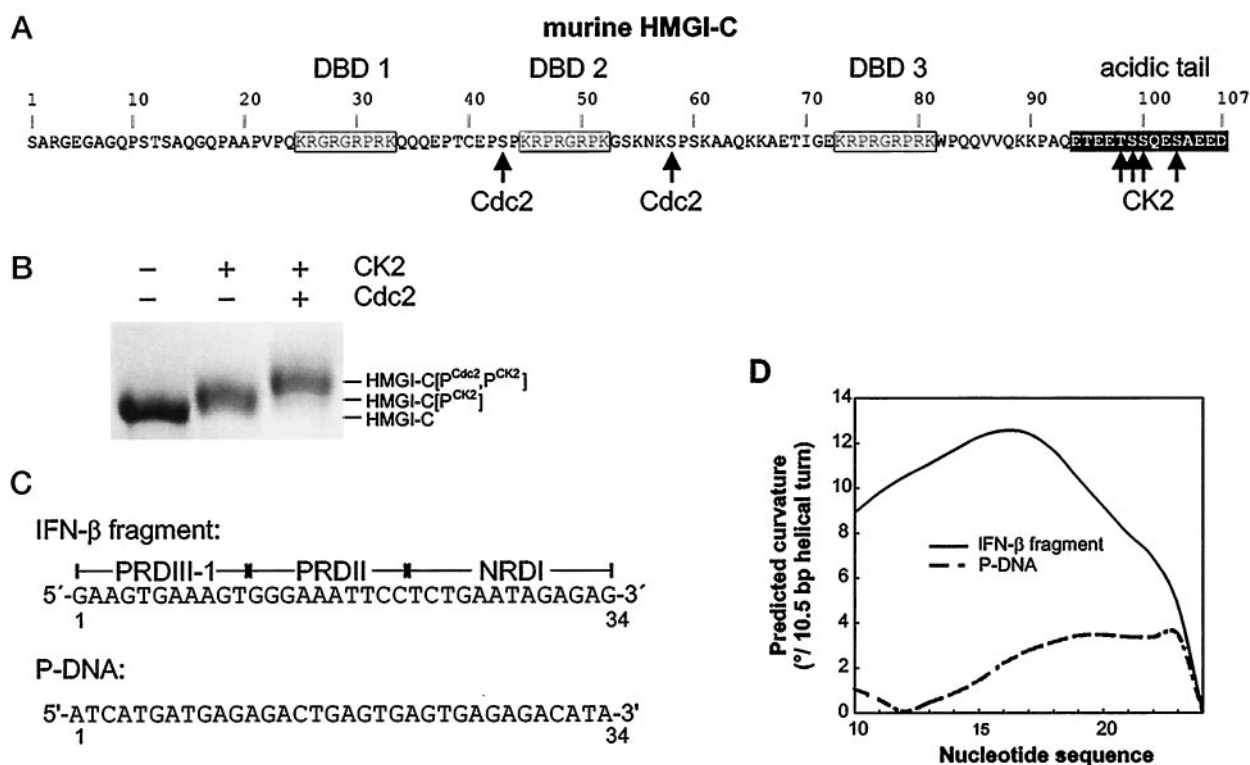
FIG. 1. *A*, primary structure of the murine HMGI-C protein. The three DNA binding domains (DBDs or so called AT-hooks) and the acidic tail of the protein are *boxed*. The phosphorylation sites of the CK2 and Cdc2 kinases are indicated by *arrows*. *B*, the bacterially expressed protein was phosphorylated *in vitro* by casein kinase 2 (HMGI-C[P^CK2]) or by both Cdc2 kinase and casein kinase 2 (HMGI-C[P^Cdc2,P^CK2]). The different phosphorylation forms were separated on a Tricine-SDS-polyacrylamide gel, and the proteins were detected by Coomassie Blue staining. *C*, top strand sequences of the 34-bp fragment of the promoter of the β-interferon gene (IFN-β; *upper sequence*) and the permutated DNA (P-DNA) with the same nucleotide composition as the IFN-β fragment (*sequence below*). *D*, predicted curvature of the IFN-β and P-DNAs according to bend.it program (available via the World Wide Web; Ref. 41) with consensus method and a window size of 10 bp.

vitro within its C-terminal region by CK2 in the presence of [^{32}P]ATP (Fig. 1*B*). Peptide mapping revealed that the protein was phosphorylated solely within its C-terminal peptide with a stoichiometry of incorporated phosphate to protein of 2:1 (data not shown). The end-labeled protein was used directly in the protein footprinting (HMGI-C[P^CK2]) analyses. To analyze the effect of Cdc2 phosphorylation on HMGI-C properties, the protein was first modified by this kinase and subsequently end-labeled by CK2 (HMGI-C[P^Cdc2,P^CK2]; Fig. 1*B*). Analysis of the peptides obtained by digestion of the phosphorylated protein with sequence specific proteinases, indicated that Cdc2 kinase phosphorylates HMGI-C at Ser-43 and Ser-58 (data not shown). These residues N- and C-terminally flank the second AT-hook of the protein (Fig. 1*A*).

Phosphorylation of the Protein by Cdc2 Kinase Changes Its Conformation and Weakens the Strength of Binding to the IFN-β Fragment—Mobility shift assays showed that phosphorylation of HMGI-C[P^CK2] by Cdc2 kinase in terms of affinity and stability weakens binding of the protein to the IFN-β fragment (Fig. 2*A*). The Cdc2 phosphorylation led to a 2–3-fold increase of the $K_{D(\text{app})}$ (Fig. 2*B*). Moreover, a substantial decrease in stability of the complex occurred upon this phosphorylation, as can be judged from the weak and diffuse appearance of the HMGI-C[P^Cdc2,P^CK2]·DNA complex (Fig. 2*A*, *right panel*). Some interaction of the protein to P-DNA was observed only at higher protein concentrations (Fig. 2*C*).

Hydroxyl-radical protein footprinting is a method allowing mapping of protein epitopes involved in macromolecular contacts (42) as well as studying changes in the protein conformation (31) with a high resolution. To get this, an extensive data analysis is necessary to transform the more slight differences from the gels, which are sometimes a little bit difficult to see for

untrained eyes, into statistically significant regions of protection or exposition. An essential step in the application of this method is preparation of a series of peptides of the analyzed protein for calibration of the gels. Limited digestions of HMGI-C[P^CK2] by proteinase Glu-C, trypsin, Arg-C, thermolysin, and iodosobenzoic acid followed by electrophoresis yielded patterns in which the individual bands could be assigned to peptides of defined length (Fig. 3*A*). Relative mobilities of the cleavage products were transformed by nonlinear regression into residue sites within the protein (Fig. 3*B*), which then allowed the alignment of hydroxyl-radical products as seen in Fig. 3*A*.

Thus, a comparison of the hydroxyl-radical digestion patterns of both protein forms without any DNA revealed that the introduction of the phosphates at position Ser-43 and Ser-58 leads to extensive changes in the cutting efficiency in distinct regions of the protein (Fig. 4*A*). For clearer documentation of the changes induced by Cdc2 phosphorylation, the intensities of the lanes were scanned (Fig. 4*B*) and the data were transformed into difference plots (Fig. 4*C*). Most significant was the increase in protection by the phosphorylation in the region between DBD 2 and 3. Moreover, an increase in exposition of regions C-terminally flanking DBD 1 and 3, respectively, was observed. The accessibility of DBDs appeared not significantly altered as a result of phosphorylation. Thus, our results suggest that in solution the protein is at least partially folded and its conformation is sensitive to posttranslational modification by Cdc2 kinase.

Binding to the Long AT Stretches of DNA Involves Three AT-hooks—Synthetic poly(dA-dT)·poly(dA-dT) is often used as a model of a binding site of HMGI(Y) proteins (12, 13, 16) since it resembles long AT stretches, which play a role in regulatory elements of some genes, where these proteins bind (43). There-
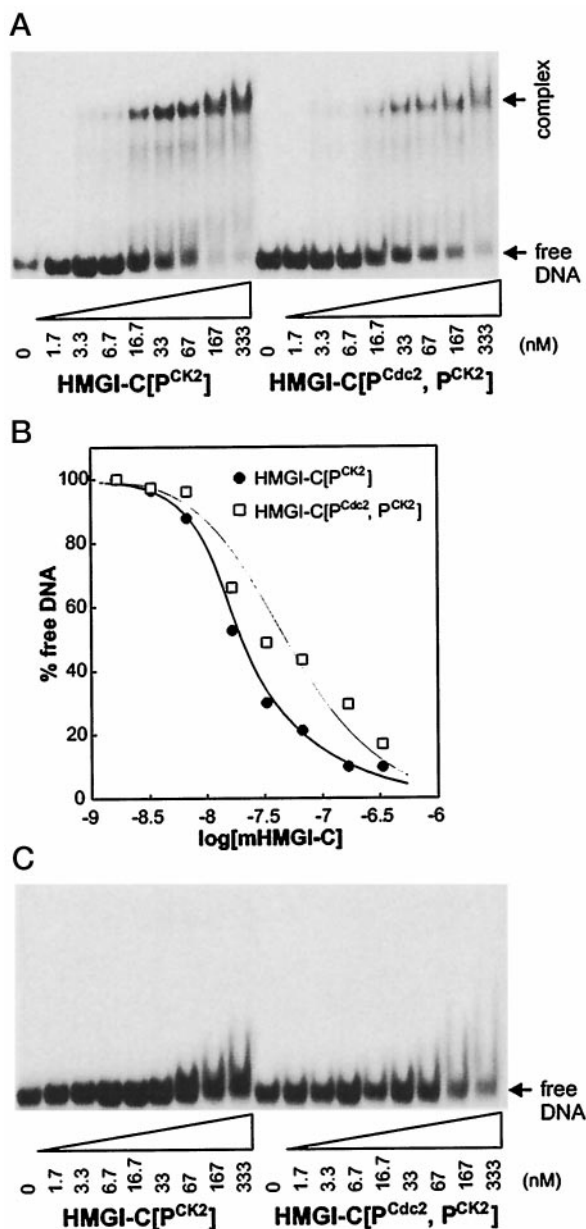
FIG. 2. **Phosphorylation by Cdc2 kinase affects binding of HMGI-C to DNA.** <1 nM ^{32}P-end-labeled IFN-β DNA (*A*) or P-DNA (*C*) was incubated with increasing concentrations of HMGI-C[P^{CK2}] (*left panels*) or HMGI-C[P^{Cdc2}, P^{CK2}] (*right panels*) and electrophoresed on 8% polyacrylamide gels. The gels were dried, and the radioactivity was scanned. *B*, quantification of the binding data from *A*. The percentage of free DNA was plotted against ligand concentration according to Carey (51). The *lines* are theoretical curves calculated from the relationship K_d = [free DNA] × [free protein]/[complexes], where $K_{d(\text{app})}$ were 20 ± 3.5 nM and 47 ± 7.4 nM for binding of HMGI-C[P^{CK2}] and HMGI-C[P^{Cdc2},P^{CK2}], respectively.

fore, a protein footprint with HMGI-C was carried out in the absence or presence of poly(dA-dT)·poly(dA-dT) (32 bp/molecule HMGI-C) (Fig. 5, *A* and *B*, *black line*). Thus, a strong protection was observed at amino acid residues 22–32, 45–54, and 76–81. Each of these regions contains a single AT-hook motif. A large portion of the protein comprising residues 56–72 was found to be highly susceptible to digestion, indicating that it was exposed to the solvent. Identical results were obtained using 10-fold higher concentrations of poly(dA-dT)·poly(dA-dT) (data not shown).

Two AT-hooks Are Involved in Contacts with β-Interferon Promoter—Footprinting in the presence of the IFN-β fragment
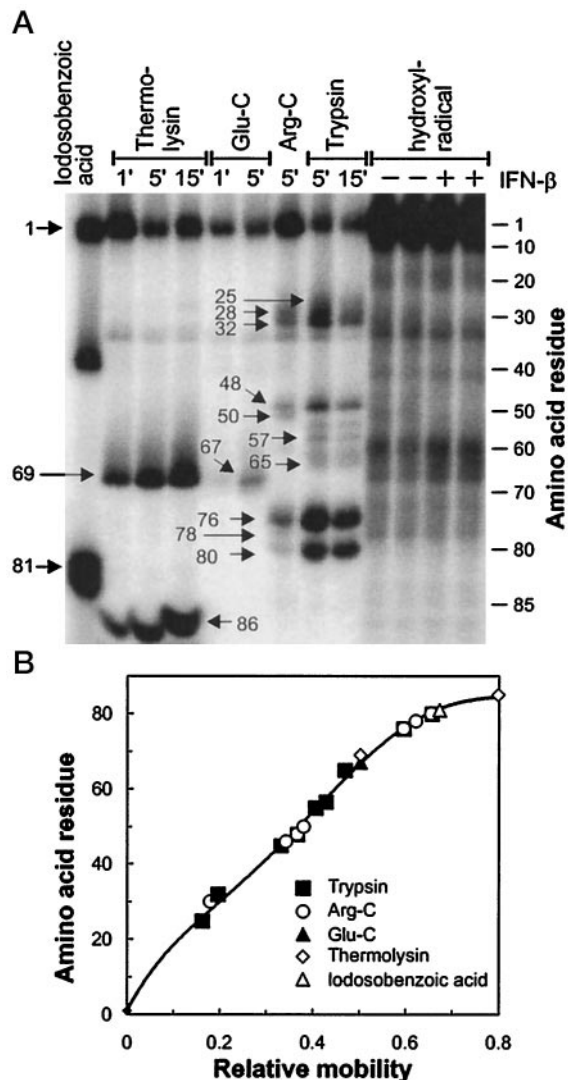

FIG. 3. **Molecular weight markers and assignment of the bands for protein footprinting.** *A*, molecular weight markers were generated by site specific cleavage of ^{32}P-end-labeled HMGI-C[P^{CK2}] with iodosobenzoic acid, thermolysin, endoproteinases Glu-C and Arg-C, and trypsin for the indicated time. *Hydroxyl-radical lanes* show peptide patterns of the protein digested with the chemical proteinase in the absence (−) or presence (+) of IFN-β DNA. *B*, plot of size of peptide markers *versus* relative mobility. Relative mobility of uncleaved HMGI-C was defined as 0 and the most rapidly migrating band of hydroxyl-radical cleavage as 1.

revealed that protein regions corresponding to residues 10–13, 23–41, and 46–53 were protected from cleavage by the chemical protease (Fig. 5*C*, *black line*). The two latter regions contain DBD 1 and 2. The region containing DBD 3 was not protected, indicating that this portion of the protein is not involved in contacts with this DNA. Similar to the HMGI-C complex with poly(dA-dT)·poly(dA-dT), a large portion of the protein comprising residues 55–78 exhibited increased susceptibility to digestion. P-DNA produced a considerably weaker pattern when compared with the result obtained in the presence of the IFN-β fragment. However, a significant but weak protection was observed at protein regions comprising DBD 1 and 2 (Fig. 5*D*, *black line*). This result emphasizes the specificity of binding of HMGI-C to the promoter fragment.

Phosphorylation by Cdc2 Kinase Reduces the Extent of Contacts with β-Interferon Promoter DNA—Phosphorylation of the HMGI-C protein by Cdc2 kinase at positions Ser-43 and Ser-58, which flank the central AT-hook motif on both sides, resulted
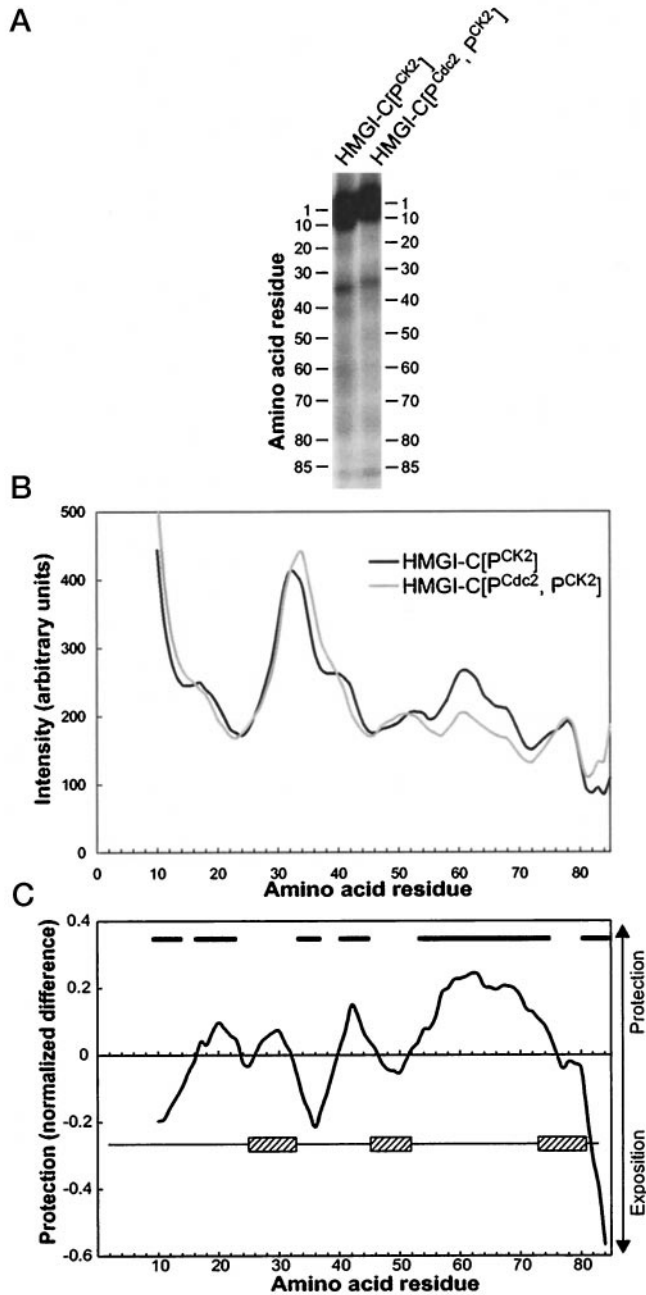
A

B

C

FIG. 4. **Conformational changes in HMGI-C[P^{CK2}] upon phosphorylation by Cdc2 kinase visualized by protein footprinting.** *A*, representative electrophoretic patterns of hydroxyl-radical digestions of the HMGI-C[P^{CK2}] and HMGI-C[P^{Cdc2},P^{CK2}] proteins from an individual experiment. *B*, plot of corrected PhosphorImager intensities. *C*, difference plot showing averaged data from six independent experiments. Positive values mean less cutting in HMGI-C[P^{Cdc2},P^{CK2}] compared with HMGI-C[P^{CK2}] at this particular position. *Bold lines* above the plot indicate regions where the observed Cdc2-induced protection or exposition was statistically significant according to a Student's *t* test (confidence level: 0.95). Schematic primary structure of the HMGI-C protein with AT-hooks (*boxes*) is shown in the *lower part* of the panel.

in dramatically altered footprinting pattern of the protein in the presence of IFN-β fragment (Fig. 5*C*). A strong decrease of the protection was observed in the region of the middle AT-hook. In contrast, the protection of the N-terminal portion of the protein and the region between residues 32 and 43 appeared to be increased. Another characteristic feature of the HMGI-C protein bound to the IFN-β fragment was a strong increase of susceptibility to digestion of the region comprising residues 55–80. In the presence of the nonspecific P-DNA,
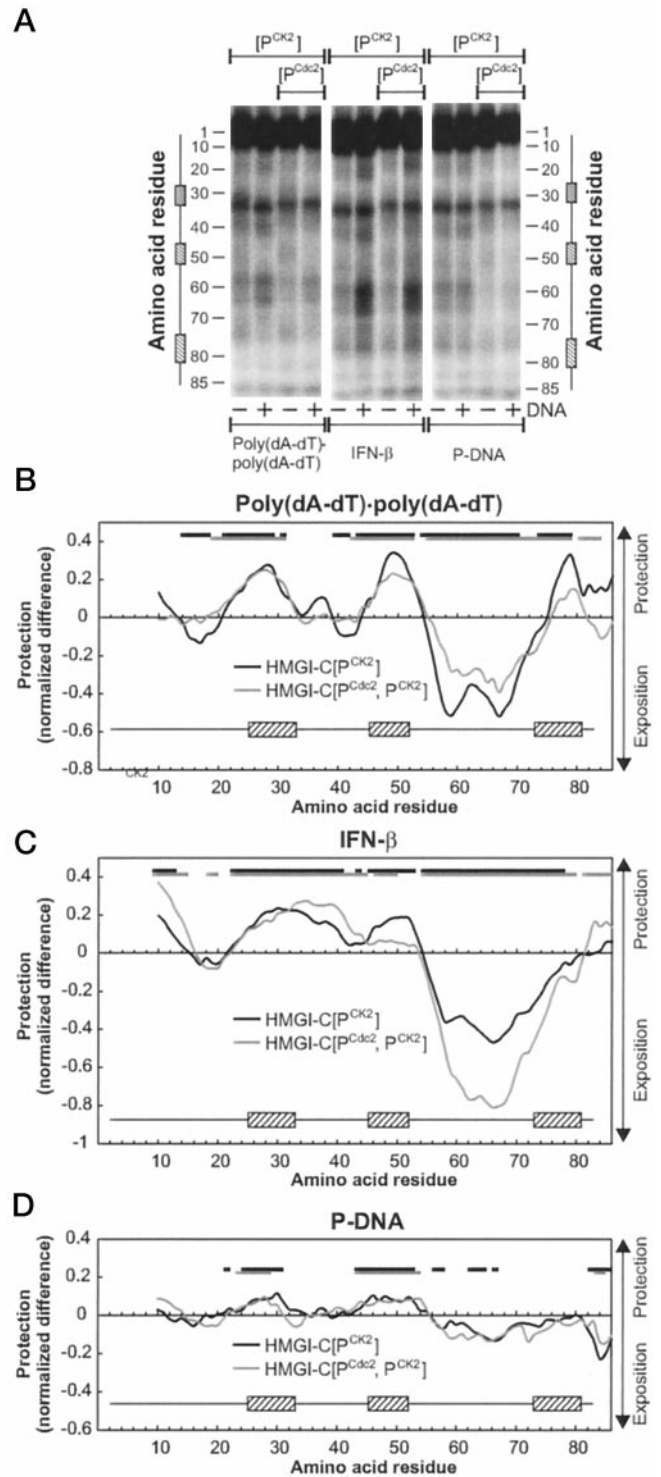
A

B

C

D

FIG. 5. **Protein footprints of poly(dA-dT)·poly(dA-dT) (*A and B*), IFN-β fragment (*A and C*) and P-DNA (*A and D*) on the end labeled HMGI-C protein.** *A*, representative electrophoretic patterns of hydroxyl-radical digestions of the HMGI-C[P^{CK2}] and HMGI-C[P^{Cdc2},P^{CK2}] proteins in the absence (−) or presence (+) of poly(dA-dT)·poly(dA-dT) (32 bp/molecule HMGI-C), IFN-β fragment (2:1 DNA/protein), or P-DNA (2:1 DNA/protein). *B–D*, difference plots showing averaged data from six independent experiments. Positive values mean less cutting in DNA-bound HMGI-C compared with unbound protein at this particular position. *Bold lines* above the plots indicate regions, where the observed protection or exposition induced by the DNA binding was statistically significant according to a Student's *t* test (confidence level: 0.95). Results of HMGI-C[P^{CK2}] and HMGI-C[P^{Cdc2},P^{CK2}] are shown in *black* and *gray lines*, respectively. Schematic primary structure of the HMGI-C protein with AT-hooks (*boxes*) are shown on *both sides* of the gels (*panel A*) and in the *lower part* of the panels (*B–D*).
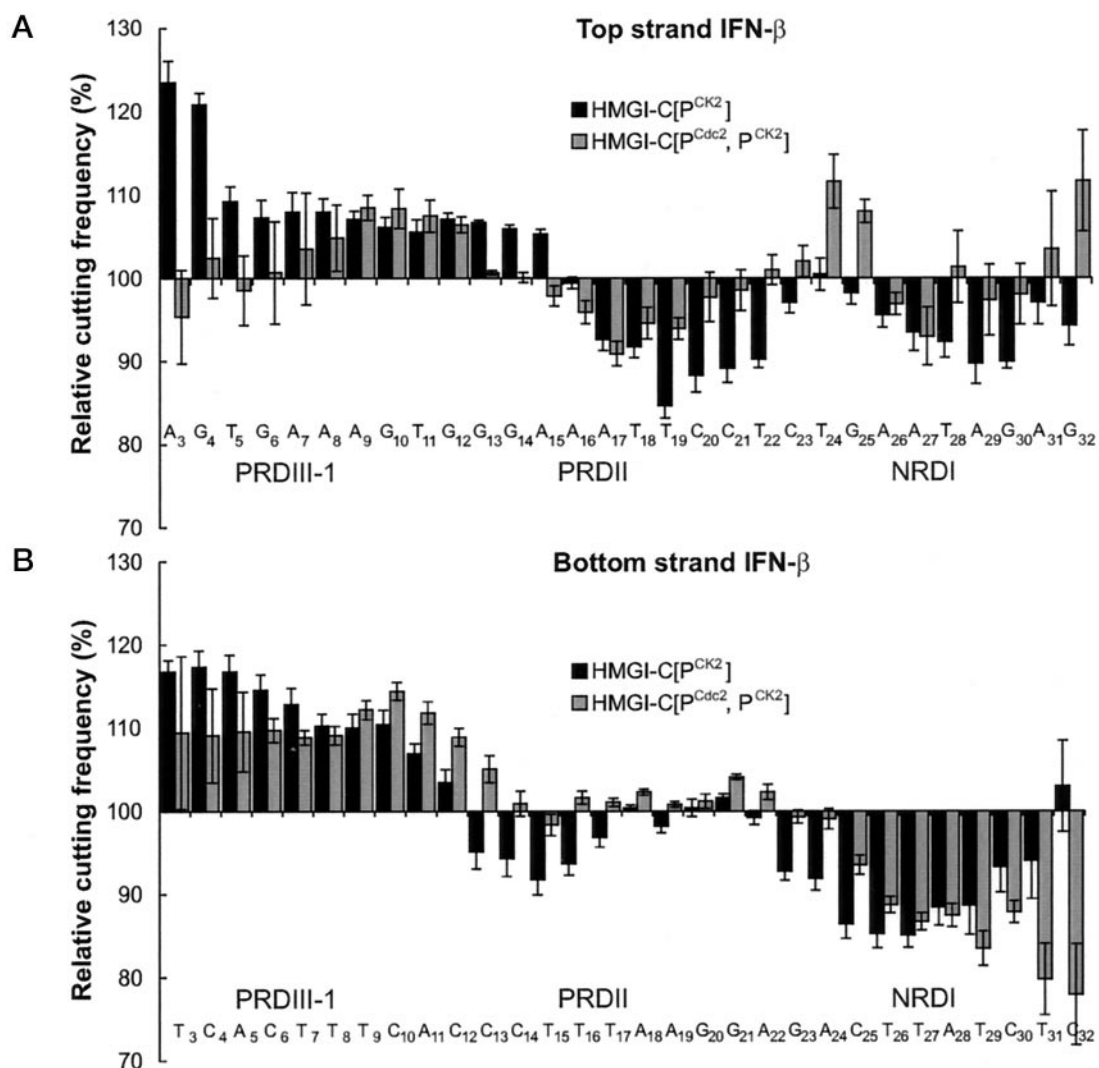
FIG. 6. **DNA footprints of the HMGI-C protein on the end-labeled IFN-β fragment.** Either the top (*A*) or the bottom (*B*) strand of the DNA were end-labeled and digested with hydroxyl-radicals in the absence or presence of HMGI-C[P^{CK2}] (*black bars*) or HMGI-C[P^{Cdc2},P^{CK2}] (*gray bars*). The reaction products were separated on a sequencing gel, 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 HMGI-C binding. The concentrations of the DNA and proteins were 30 and 60 nM, respectively. The presented results are mean values from four independent experiments. *Thin bars* indicate ± standard deviation.

weak and mostly non-significant changes in the footprinting pattern were observed (Fig. 5*D*), suggesting weak and unspecific interaction between this DNA and the protein, which is not sensitive to phosphorylation by Cdc2 kinase. Footprints with Cdc2 kinase phosphorylated HMGI-C in the presence of poly(dA-dT)·poly(dA-dT) were very similar to those obtained for the protein that is not phosphorylated by Cdc2; however, some weakening of the protection was observed in the regions containing DBD 2 and 3 (Fig. 5*B*).

HMGI-C Binds to PRDII and NRDI Elements of the Promoter—To obtain information on the DNA regions that interact with the protein, we also carried out DNA footprinting experiments and methylation interference assays. Binding of HMGI-C[P^{CK2}] resulted in the protection of the IFN-β promoter at bases 17–23 and 25–32 of the top strand (Fig. 6*A*, *black bars*) and bases 13–17, 19, and 22–31 of the bottom strand (Fig. 6*B*, *black bars*). The protected regions correspond to the PRDII and NRDI sites. The third AT-rich tract of the 34-bp DNA, the PRDIII-1, was not protected, suggesting that it is not involved in the interaction. On both strands the observed maxima of protection within the PRDII and NDRI elements are 10–11 bases apart (Fig. 6, *A* and *B*, *black bars*). Phosphorylation of the

protein by Cdc2 kinase resulted in a general decrease of the protection on the top strand and led to a slight shift of the binding toward the 5′ end both within PRDII and NRDI elements (Fig. 6*A*). On the bottom strand contacts within the PRDII site were totally abolished, whereas at the positions within the NRDI both qualitative and quantitative changes were observed (Fig. 6*B*). In this region the strongest protection was observed at T29, T31 and C32. Thus, within NRDI element of the bottom strand Cdc2 phosphorylation results in sliding of the protection maxima by several bases in direction of their 5′ ends (Fig. 6*B*).

Methylation of the purines A16-A17 and G25, A26, A27, A29, G30, and A31 of the top strand interfered with binding of the HMGI-C[P^{CK2}] protein (Fig. 7*A*, compare *lanes 2* and *3*). The interference with modified adenines is in a good agreement with the expected interaction of the AT-hooks within the minor grooves. In addition, interference by modification at G25 and G30 may suggest that regions flanking the AT-hook bound to NRDI interact with the element through the major groove. In contrast, methylation of the purines interfered much less with the binding of the protein when phosphorylated by Cdc2 kinase (Fig. 7*A*, *lanes 3* and *4*), suggesting that the phosphorylated
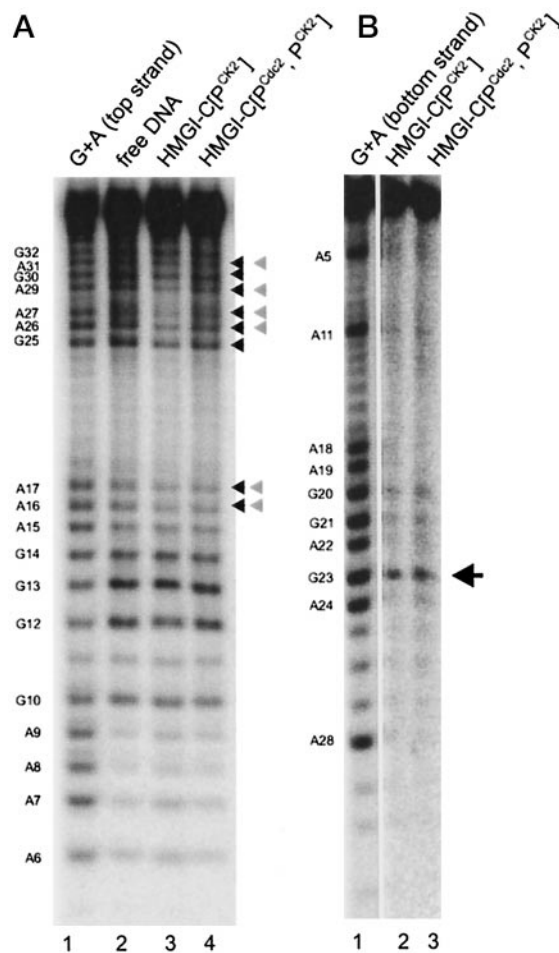
FIG. 7. **Methylation interference assay (*A*) and determination of the position of Cys-40 (*B*) in the protein complex with the IFN-β fragment.** *A*, the top strand was treated with dimethyl sulfate and bound to HMGI-C[P^{CK2}] or HMGI-C[P^{Cdc2},P^{CK2}]. The complexes were isolated from preparative mobility shift gels, cleaved with piperidine, and analyzed on sequencing gels. The *arrowheads* indicate the bases that interfere with binding of HMGI-C[P^{CK2}] (*black*) and HMGI-C[P^{Cdc2},P^{CK2}] (*gray*). Interference with the Cdc2-phosphorylated protein was much weaker or disappeared at all positions compared with HMGI-C that was not Cdc2-phosphorylated. *B*, determination of the nucleotide(s) of the bottom strand at which cross-linking of HMGI-C[P^{CK2}] and HMGI-C[P^{Cdc2},P^{CK2}] derivatives occurred. The proteins were selectively derivatized at Cys-40 with 4-azidophenacyl bromide, bound to ^{32}P-end-labeled IFN-β fragment, and UV-irradiated (λ > 290 nm). The isolated complexes were digested at modified bases in alkali/heat reaction and separated on sequencing gels. The main cross-linking position at G23 of the bottom strand is labeled with an *arrow*. No cross-linking of the derivatives with the top strand was observed (data not shown)

protein does not bind tightly within the minor groove of the AT-tracts.

Localization of the Cys-40 Residue in the Complex—The residue Cys-40 lies between DBD 1 and 2 of HMGI-C, which are primarily involved in the protein binding to DNA. To obtain more precise information on the location of this residue with respect to the IFN-β fragment, we applied the site specific protein-DNA photocross-linking method (36). The method involves modification of the thiol moiety azidophenacyl bromide, formation of the derivatized protein-DNA complex, UV irradiation, and determination of the nucleotides at which cross-linking occurs. Results presented in Fig. 7*B* demonstrate that the cross-linking of the protein-azidophenacyl-derivative occurred predominantly at G23 of the bottom strand of the DNA, indicating that the Cys-40 residue occupies a position adjacent to the DNA between PRDII and NRDI elements (Fig. 7*B*, *lane*

2). No cross-linking with the bases of the top strand was observed (data not shown). A similar result was obtained with the azidophenacyl-derivative of the protein phosphorylated at Ser-43 and Ser-58 (Fig. 7*B*, *lane 3*), indicating that phosphorylation by Cdc2 kinase did not influence intermolecular vicinity between Cys-40 and G23.

DISCUSSION

Results obtained in this study allow a deeper insight to the nature of the interaction between HMGI(Y) type proteins and regulatory elements of the IFN-β gene or pure AT-DNA stretches. Our results extend the picture of the complex of the AT-hook domain with DNA that was obtained by NMR spectroscopic studies (17). We provide the first description of the organization of a complex of HMGI-C, a member of the HMGI(Y) protein family, and a fragment of the promoter of the β-interferon gene, based on experiments employing the entire protein carrying posttranslational modifications, that are characteristic for its native form.

Protein footprinting experiments revealed that, in the presence of unspecific AT-rich DNA, all three AT-hooks present in the protein are involved in contacts with DNA, whereas, in the presence of the promoter of the β-interferon gene, only the first two binding domains are directly involved in binding. A similar situation was described previously for the *Chironomus* HMGI protein (16); however, the insect protein binds to the promoter of the β-interferon gene using the second and the third motif. Interestingly, in both cHMGI and HMGI-C, the distance between the centers of those AT-hooks that bind to the promoter is exactly 20 residues. In mammalian HMGI, the distances between the first and the second and the second and the third motifs are 32 and 26 residues, respectively. In this case also, the last both AT-hooks are involved in the binding to the IFN-β promoter (15), indicating that this distance of AT-hooks is a prerequisite for interaction with DNA of this type.

In the complex with the IFN-β fragment, the third AT-hook of HMGI-C is not involved in contacts with DNA. Moreover, in the complex, this region and a portion of the protein comprising residues that are N-terminally adjacent to it are strongly exposed to solvent. Therefore, this portion of protein may be responsible for protein-protein contacts in assembly of higher order complexes, as those of the enhanceosome. The propensity of AT-hooks to interact with other proteins was previously demonstrated for nuclear factor Y (NF-Y) and HMGI(Y) (44) and serum-response factor (SRF) and HMGI(Y) (45). More recently, Yie *et al*. (46) have suggested that completion of the enhanceosome assembly requires interaction of DNA-bound HMGI(Y) with transcriptional activators. Thus, a HMGI(Y) protein bound to a promoter would facilitate binding of the activators through the AT-hook, which is not involved in DNA binding. Binding of proteins of the HMGI(Y) family to pure AT-tracts results in masking of all three AT-hooks (Ref. 16, and this work) and thus probably inhibits binding to the activators. This property of the HMGI(Y) protein family appears to be important in view of their association with DNA of constitutive chromatin (6, 47), where recruitment of transcriptional activators is not desired.

DNA footprinting and methylation interference assays clearly demonstrate that HMGI-C binds to the PRDII and NRDI elements. Binding of the HMGI protein to this DNA occurs also at these elements (15), suggesting that both proteins exhibit similar specificity and probably are able to exert similar function. The quantitative analysis of the footprinting data revealed that the sites protected in the complex match very well with the positions of the sugar-phosphate backbone that contact the central AT-hook of human HMGI (17). The primary structure of this AT-hook is identical in HMGI and
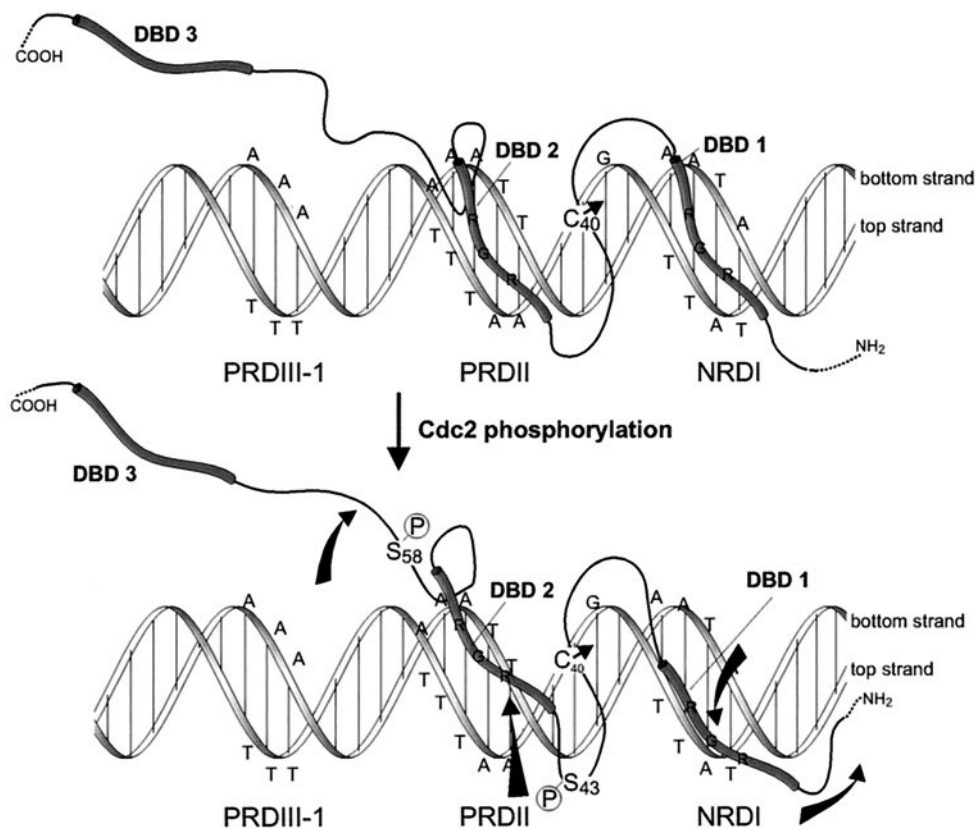
FIG. 8. **Model of DNA binding by HMGI-C within the IFN-β fragment and its modulations upon Cdc2 phosphorylation.** DBD 1 and 2 of the HMGI-C protein bind to the NRDI and PRDII elements of the IFN-β promoter, respectively. After phosphorylation of the protein at Ser-43 and Ser-58 by Cdc2 kinase multiple contacts of DBDs, especially with the bases, are impaired and the protein binds to DNA in a different way, extending the contacts to the sugar-phosphate backbone. Major changes in the complex are indicated by *arrows*.

HMGI-C. The maxima of protection correspond to positions where the AT-hook enters and exits the minor groove, *e.g.* in the vicinity of T17 and T21 of the upper and bottom strands, respectively. The higher intensity of protection within the upper strand of PRDII appears to reflect the higher extent of contacts between DBD 2 and the upper strand in comparison to the bottom strand (17). On the basis of the intensity of protection within the NRDI element, it appears that the contacts between the protein and DNA at this site are more extensive than those observed for the PRDII element. As the binding of the first AT-hook to the promoter of the β-interferon gene appears to involve a region that flanks it C-terminally (residues 33–41), it seems that this part of HMGI-C is responsible for contacts with NRDI. A short stretch of three glutamine residues is present in the region that flanks DBD 1 C-terminally. Glutamine side chain has strong hydrogen binding potential and is often involved in contacting A and G bases within major and minor grooves, respectively. Therefore, it is possible that these residues are integral elements of this DBD. To distinguish it from the previously defined DBDs of HMGI(Y) of type I and II (17), we propose to designate it DBD of type III.

Activation of Cdc2 kinase during G_2 phase of the cell cycle is essential for the onset of mitosis in eukaryotic cells. A number of nuclear proteins become phosphorylated by this enzyme. This includes nuclear lamins, nucleolin, histone H1, HMGI(Y), and many other proteins. During cell division HMGI(Y) proteins remain associated with condensed mitotic chromosomes (6, 48) despite their mitotic phosphorylation (20, 21).

The HMGI-C protein contains two sites that are substrates for Cdc2 kinase, Ser-43 and Ser-58. Phosphorylation at these sites results in a 4-fold reduction in the strength of binding to IFN-β promoter. Previous studies, in which the effect of phos-

phorylation on DNA binding by HMGI was studied, revealed that differences in binding affinity between phospho- and dephospho- forms of this protein are salt-dependent (21). An increase in salt concentration weakens much more severely the binding of the Cdc2-phosphorylated protein than that of the dephosphorylated one. This suggests that the contribution of ionic interactions in the complex of the phosphorylated HMGI with DNA is higher than in the case of the complex with dephosphorylated protein. Our results demonstrated dramatic changes in the nature of the observed contacts. This includes mainly impairment of a number of contacts between the second AT-hook and the PRDII element and an apparent strengthening of binding of the first AT-hook to the NRDI element on the bottom strand (Fig. 6). The weakening of the binding of the phosphorylated protein to PRDII seems to be partially counterbalanced by additional contacts at the bottom strand of the NRDI element. The observed alterations upon modification by Cdc2 are best accommodated in the model depicted in Fig. 8. It appears that phosphorylation leads to an at least partial *derailing* of the DBDs from the minor groove, which may result in additional ionic contacts with the sugar-phosphate backbone. *In vivo*, phosphorylation by Cdc2 kinase may contribute to the disassembly of promoter complexes in cells entering mitosis. Strong perturbation of the complex of HMGI-C with the β-interferon promoter upon phosphorylation of the protein by Cdc2 kinase, observed in this study by footprinting techniques, is probably accompanied by changes in the conformation of the DNA. Further studies, in which DNA bending can be monitored either by circular permutation assay (40) or by means of fluorescence spectroscopy (49, 50), would allow us to extend our knowledge of the action of the HMGI-C in β-interferon promoter complex.

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**Architecture of High Mobility Group Protein I-C·DNA Complex and Its
Perturbation upon Phosphorylation by Cdc2 Kinase**

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