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Consecutive Steps of Phosphorylation Affect Conformation and DNA Binding of the *Chironomus* High Mobility Group A Protein*

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The high mobility group (HMG) proteins of the AT-hook family (HMGA) lie downstream in regulatory networks with protein kinase C, Cdc2 kinase, MAP kinase, and casein kinase 2 (CK2) as final effectors. In the cells of the midge *Chironomus*, almost all of the HMGA protein (cHMGA) is phosphorylated by CK2 at two adjacent sites. 40% of the protein population is additionally modified by MAP kinase. Using spectroscopic and protein footprinting techniques, we analyzed how individual and consecutive steps of phosphorylation change the conformation of an HMGA protein and affect its contacts with poly(dA-dT)·poly(dA-dT) and a fragment of the interferon-β promoter. We demonstrate that phosphorylation of cHMGA by CK2 alters its conformation and modulates its DNA binding properties such that a subsequent phosphorylation by Cdc2 kinase changes the organization of the protein-DNA complex. In contrast, consecutive phosphorylation by MAP kinase, which results in a dramatic change in cHMGA conformation, has no direct effect on the complex. Because the phosphorylation of the HMGA proteins attenuates binding affinity and reduces the extent of contacts between the DNA and protein, it is likely that this process mirrors the dynamics and diversity of regulatory processes in chromatin.

High mobility group (HMG)[1] proteins are nonhistone proteins that are thought to play various roles in the assembly of chromatin and the regulation of transcription (for a review see Refs. 1 and 2). This group comprises three families of structurally unrelated proteins: HMGB,[2] HMGN, and HMGA (previously HMG-1/2, HMG-14/17 and HMGI/Y; for a review see Refs. 3 and 4). The presence of 2–15 AT-hooks, the putative DNA binding domains (DBD), is common for the HMGA family (for comparison of multiple AT-hook-containing proteins see Ref. 5). The AT-hooks bind within the minor groove of AT-rich DNA (6). Simultaneous binding of two or more of these domains increases the strength of interaction of these proteins with DNA (7–9). Mammalian HMGA1a, HMGA1b, HMGA2, and insect cHMGA (formerly cHMGI) have three DBDs. Depending upon the type of DNA examined, all or only two of these DBDs are directly involved in binding (10). DBD2 plays a central role in the organization of the protein-DNA complexes (9–14), whereas binding of the two other DBDs to DNA depends on the DNA sequence and conformation (9, 10, 14). Comparative analyses of binding of the HMGA proteins to the interferon-β promoter have shown that each of the four analyzed HMGA proteins binds in a specific manner (14). This supports the idea that each of the HMGA proteins has an individual function, *e.g.* in the regulation of specific sets of genes (15, 16).

The proteins of the HMGA family are phosphorylated *in vivo* by Cdc2 kinase (17, 18), MAP kinase (19), and CK2 kinase (20, 21). In addition, efficient phosphorylation of these proteins by PKC has also been demonstrated (19, 22, 23). These posttranslational modifications attenuate the DNA binding affinities of mammalian HMGA1a (17, 18, 22, 24), HMGA1b (14), murine HMGA2 (12), and insect cHMGA (19). Structural studies revealed that phosphorylation of HMGA2 protein by Cdc2 kinase impairs DNA binding of DBD2 (12), whereas phosphorylation of HMGA1b by this kinase mainly affects the contacts of DBD1 with DNA (14).

The cHMGA protein from *Chironomus tentans* is the only characterized HMGA protein originating from a non-mammalian species. Like its mammalian counterparts, it has three AT-hook domains and a C-terminal stretch of mainly acidic amino acid residues (7). Moreover, mammalian and insect proteins are phosphorylated by the same kinases. In this work we studied the consequences of phosphorylation of cHMGA on the conformation of the protein and its interaction with DNA. We show that phosphorylation of cHMGA by CK2, Cdc2 kinases, and MAP kinase affects the conformation of the protein. Phosphorylation of the protein by CK2 and Cdc2 kinase impairs DNA binding of the AT-hook domains located close to the N and

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[1] The abbreviations used are: HMG, high mobility group; DBD, DNA binding domain; cHMGA, *Chironomus* HMGA protein; MAP, mitogen-activated protein; MAPK, MAP kinase; CK2, casein kinase 2; Cdc2, cyclin-dependent cell cycle 2; PKC, protein kinase C; HPLC, high pressure liquid chromatography; MOPS, 4-morpholinepropanesulfonic acid; acrylodan, 6-acryloyl-2-dimethylaminonaphthalene; IFNβ, interferon-β; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

[2] The nomenclature of the mammalian HMG proteins has been revised recently; see the HMG protein home page. Consequently, we also renamed the cHMGI protein of *Chironomus* "cHMGA." In this paper we use the new nomenclature of HMG proteins (the old nomenclature is given in parentheses). HMGA (HMGI/Y family); HMGA1a (HMGI), HMGA1b (HMGY); HMGA2 (HMGI-C); HMGB (HMG-1/2 family); HMGN (HMG-14/17 family); cHMGA (cHMGI).

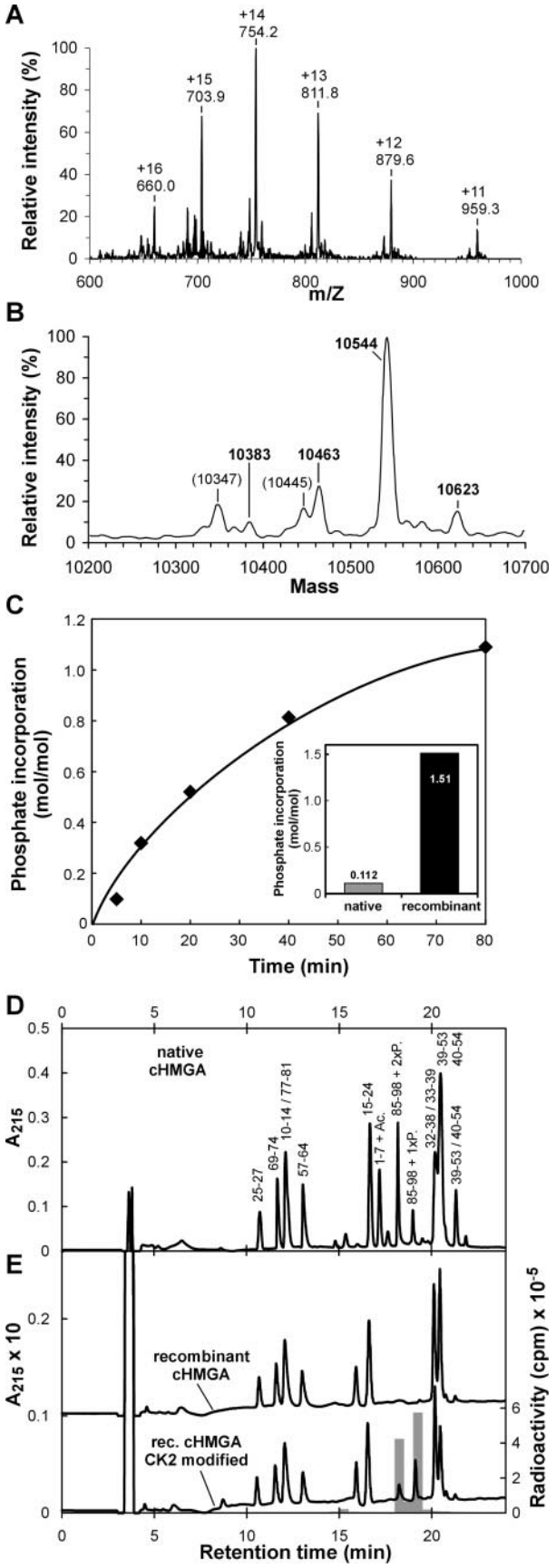

FIG. 1. **The phosphorylation at residues 96 and 97 is the main posttranslational modification in the cHMGA protein and is caused by CK2.** *A*, electrospray ionization mass spectrum obtained for a mixture of native cHMGA. *B*, a deconvoluted spectrum of *A* shows the presence of four components with M_r of 10,383, 10,463, 10,544, and 10,623, differing by ~80 units (indicated by *bold numbers*). The value of 10,383.0 is in agreement with the calculated M_r of acetylated cHMGA. The identity of two other signals is unknown (M_r values are given in *parentheses*). *C*, time course of the *in vitro* phosphorylation of recombinant cHMGA. The proteins were phosphorylated using recombinant human CK2 in the presence of [γ-^{32}P]ATP. At the indicated times, the reactions were terminated by precipitation with 33% CCl_3COOH, and the incorporated radioactivity was measured. *Inset,* a 2.5-h reaction resulted in an incorporation of 1.51 and 0.112 mol of phosphate per 1 mol of recombinant and native protein, respectively. *D*, mapping of the phosphorylation sites in the main fraction of the native cHMGA. ~50 μg of the native protein were digested with 5 μg of trypsin for 3 h, and the digestion products were separated on a reverse-phase C_{18} column. The identity of the individual peptides was determined by mass spectrometry (Table I). *E*, comparative mapping of the *in vitro* phosphorylation sites of CK2. Either radioactively phosphorylated or nonphosphorylated recombinant cHMGA was digested with trypsin and chromatographed as described above. Fractions carrying the radioactive label are indicated by *shaded areas*.

C termini of the protein. In contrast, phosphorylation of Ser-22 by MAP kinase does not affect protein binding to DNA and appears to have another function, *e.g.* modulation of protein-protein contacts.

EXPERIMENTAL PROCEDURES

Construction and Expression of cHMGA Mutants—Three double mutants of the cHMGA protein, in which two residues were mutated to Cys and Trp, respectively, were constructed by means of a polymerase chain reaction. The cysteine and tryptophan residues were introduced at positions 3 and 94 ((Cys-3)cHMGA), 15 and 1 ((Cys-15)cHMGA), and 94 and 1 ((Cys-94)cHMGA; see Fig. 2). The constructs were cloned into the expression vector pET3a, and the proteins were overexpressed in BL 21 (DE3) cells as described (25–27).

Protein Extraction and Purification—The proteins were isolated by extraction with 5% (v/v) $HClO_4$ in three freezing-thawing cycles from cultured *Chironomus* cells or from bacteria expressing cHMGA or its mutants (7). The cell supernatants were acidified with HCl to 0.35 M, and proteins were precipitated with 6 volumes of acetone and dried. Crude extracts were separated on a cation-exchange column (Poros SP20, 4.6 × 100 mm, PerSeptive Biosystems) using a 0.3–1 M NaCl gradient in 25 mM sodium borate, pH 9.4. To the fraction containing the mutated proteins 1 M dithiothreitol was added to the final concentration of 50 mM. After a 30-min incubation at 20 °C the samples were concentrated and desalted on Sep-Pack Plus C_{18} cartridges (Waters) using 70% CH_3CN, 0.1% CF_3COOH in H_2O as the eluent. Finally, the proteins were chromatographed on a reverse-phase C_{18} Zorbax SB-300 column using a linear CH_3CN gradient in 0.1% CF_3COOH in H_2O as described previously (25). The Trp residues introduced into the mutants allowed quantification of the proteins by spectrophotometry using an absorption coefficient for tryptophan of 5,500 M^{-1} cm^{-1} at 280 nm. The wild type cHMGA was quantified on Coomassie Blue R-stained polyacrylamide gels using one of the cHMGA mutants as a standard.

Phosphorylation of the Proteins—50 μg of the native or recombinant cHMGA 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 (50 mM Tris/HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM ethylene glycol-bis(β-aminoethyl ether) tetraacetic acid, pH 7.5). 50 μg of purified cHMGA protein or Cdc2-phosphorylated cHMGA were phosphorylated at 37 °C with 500 units of recombinant human CK2 (New England Biolabs Inc.) for 2.5 h in the presence of 200 μM ATP in 50 μl of CK2 buffer (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 were added to the reaction mixture. The reaction products were separated by reversed-phase HPLC. The cHMGA[P^{MAPK},P^{CK2}] form of cHMGA was isolated from *Chironomus* cells as described previously (19).

Modification of the Mutants of the cHMGA Protein with Acrylodan—50 μM protein in 25 mM MOPS/NaOH, 150 mM NaCl, pH 7.0 was incubated with a 3–4-fold molar excess of acrylodan (6-acryloyl-2-dimethylaminonaphthalene; Molecular Probes). The reaction was carried out at 4 °C for 3–5 h, and the reaction products were separated by reverse-phase HPLC as described above. To verify that the proteins were phosphorylated and modified by acrylodan at the desired sites, we digested the proteins with trypsin in 0.1 M Tris/HCl, pH 7.5 at 37 °C for 3 h. Peptides were resolved and identified by reverse-phase C_{18} chromatography as described previously (19). The use of the diode array

TABLE I
Mass spectrometric analysis of the native cHMGA protein and its peptides obtained by tryptic digestion

Elution time[a]	Observed M_r[b]	Sequence position	Calculated M_r[b,c]	Concluded posttranslational modification
min				
	10,383		10,340	1 acetylation
	10,463	Entire protein	10,340	1 phosphorylation + 1 acetylation
	10,544	1–98	10,340	2 phosphorylations + 1 acetylation
	10,623		10,340	3 phosphorylations + 1 acetylation
10.7	287.5	25–27	288.3	None
	385.5	65–68	385.5	None
11.6	614.4	69–74	614.7	None
12.1	527.5	10–14 and/or 77–81	527.6	None
13.0	843.8	57–64	843.9	None
16.6	1057.2	12–24	1057.2	None
17.2	801.7	1–7	759.9	Acetylation
18.2	1653.4	85–98	1493.4	Double phosphorylation
19.0	1573.9	85–98	1493.4	Single phosphorylation
20.2	827.6	32–38 and/or 33–39	828.0	None
20.5	1599.2	39–53 or 40–54	1598.8	None
21.3	1599.2	39–53 or 40–54	1598.8	None

[a] Peptides were separated by reverse-phase HPLC (Fig. 1*D*) as described under "Experimental Procedures."
[b] The M_r values were calculated using monoisotopic masses for peptides less than 1,000, whereas for larger peptides average values were used.
[c] The values were calculated based on known protein sequence (7).

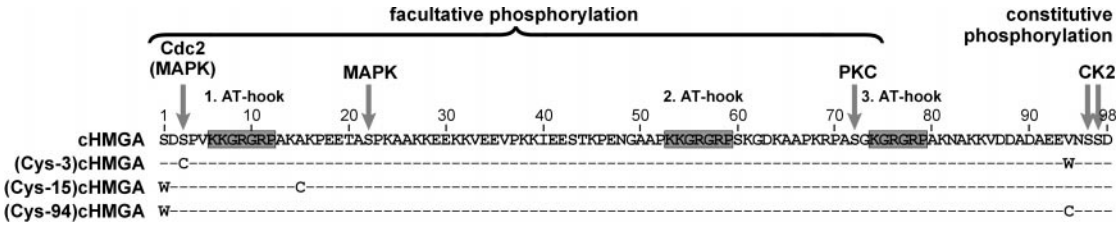

FIG. 2. **The primary structures of the cHMGA protein and its mutants.** The sites phosphorylated by Cdc2 kinase (*Cdc2*), MAPK, PKC, and CK2 within cHMGA are indicated by *arrows*. The putative DBDs (or AT-hooks) are *boxed*.

spectral detector enabled easy identification of peptides containing tryptophan or acrylodan-modified cysteine residues.

DNA and Oligonucleotides—Synthetic linear poly(dA-dT)·poly(dA-dT) DNA was obtained from Amersham Pharmacia Biotech. The approximate average length of this DNA was 5000 base pairs. The 34-base pair fragment of the promoter of the IFNβ gene containing the positive regulatory domains III-1 and II and the negative regulatory domain I was prepared from synthetic oligonucleotides (10).

Hydroxyl Radical Protein Footprinting—The protein footprinting reactions and quantitative analyses of the products were performed as described previously (10, 12–14, 28, 29). Briefly, 10 pmol of the radioactively end-labeled protein (10–20 kcpm) were digested at room temperature in the presence or absence of DNA in a total volume of 10 μl containing 180 mM NaCl and 10 mM MOPS buffer, pH 7.2. 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 $(NH_4)_2Fe(II)(SO_4)_2$, (ii) 0.2 M sodium ascorbate, and (iii) 0.375% (v/v) H_2O_2. Reactions were stopped after 30 min by the addition of 3.3 μl of 4× concentrated 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 Tricine-SDS-polyacrylamide gels and analyzed by phosphorimaging as described previously (10). Size markers were generated by limited digestion of 10 pmol of end-labeled HMG protein by trypsin, thermolysin, proteinase Arg-C, or proteinase Glu-C in a 10-μl reaction volume. 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 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. Cleavage with proteinase Glu-C was carried out in the presence of 5 or 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 or 0.5 μg 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× concentrated SDS sample buffer containing 20 mM EDTA.

Limited Proteolytic Digestion—A mixture of native phosphoforms and the recombinant protein (molar ratio of 1:1:1), each labeled at Ser-3 by Cdc2 modification, was digested with endoproteinase Glu-C in 25 mM sodium phosphate, pH 7.8 and 180 mM NaCl at 20 °C. The protein to enzyme ratio was 50:1 (mol/mol). Reactions were terminated by addition of an equal volume of 10 M urea solution containing 5% (v/v) acetic

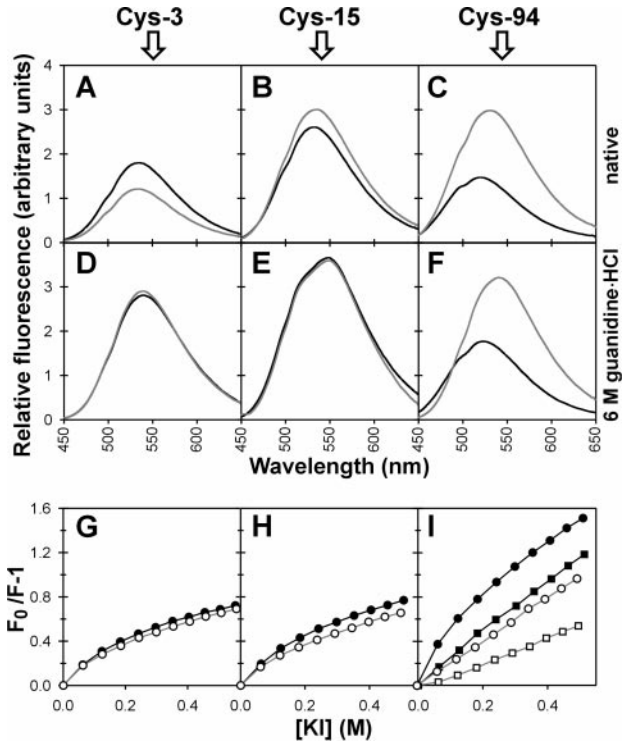

FIG. 3. **Fluorescence spectroscopy reveals differences in conformation between nonphosphorylated and CK2-phosphorylated protein.** *A–F*, fluorescence emission spectra of nonphosphorylated (*black lines*) and phosphorylated (*gray lines*) single cysteine mutants modified with acrylodan in the absence (*A–C*) and presence (*D–F*) of 6 M guanidine·HCl. The excitation wavelength was 391 nm. *G–I*, Stern-Volmer plots for fluorescence emission quenching by KI measured at 530 nm. *Closed symbols*, non-phosphorylated proteins; *open symbols*, CK2-phosphorylated proteins. The squares in *I* represent quenching in the presence of 6 M guanidine·HCl.

acid, 4% (v/v) β-mercaptoethanol, 10 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, and 0.2 mM *N*α-tosyl-L-lysine chloromethane. Reaction products were separated on 15% polyacrylamide urea-acetic acid-Triton X-100 gels (30). Gels were scanned with a PhosphorImager (Molecular Dynamics), and the intensities of the bands were quantified.

Mass Spectra—Mass spectra were recorded on a Finnigan MAT TSQ 700 triple-stage quadrupole mass spectrometer equipped with an electrospray ion source. Samples were typically dissolved in a methanol/water/acetic acid (47:48:5, v/v/v) solution at a concentration of 50 pmol/μl and introduced into the electrospray needle by mechanical infusion through a microsyringe at a flow rate of 1 μl/min. A potential difference of 4.5 kV was applied between the electrospray needle. Nitrogen gas was used to evaporate the solvent from the charged droplets. At least 20 scans were averaged to obtain each spectrum. The resulting spectra were transformed using a BioWorks software package (Finnigan).

Fluorescence Measurements—Emission spectra with or without quenching were taken on an SLM-Aminco-Bowman series 2 luminescence spectrometer in 10 mM MOPS/NaOH, pH 7.2, 100 mM sodium acetate, 100 μg/ml bovine serum albumin. Measurements were corrected for buffer background, dilution, and instrumental factors. Excitation was at 391 nm. Emission spectra were recorded using 4- and 2-nm slits for excitation and emission, respectively. The titrations with KI were performed using 2- and 16-nm slits for excitation and emission (530 nm), respectively.

Circular Dichroism Measurements—CD spectra of 20–50 μM (Cys-94)cHMGA protein in 10 mM sodium phosphate, pH 7.5 were recorded at a scanning speed of 50 nm/min from 190–280 nm in cuvettes of 1-mm path length with a Jasco 720 spectropolarimeter using a 1-nm bandwidth. For each sample three CD spectra were averaged to improve the signal-to-noise ratio. Prior to each CD measurement the protein concentration of the sample in the cuvette was determined in a Cary 100 spectrophotometer (Varian Inc.) using an absorption coefficient of $\epsilon_{206} = 268{,}550\ M^{-1}\ cm^{-1}$. Jasco Secondary Structure Estimation software (version 1.00.00, 1998) was used to estimate the amount of secondary structure elements.

Lifetime Measurements—Fluorescence lifetimes of acrylodan-labeled proteins were determined by time-correlated single photon counting. The excitation was at 405 nm with a pulsed diode laser (IBH, Glasgow, Scotland) operating at 1 MHz, and emission was collected using a 520-nm interference filter. The fluorescence was detected by an integrated photon detection module (TBX-04, IBH) containing a photomultiplier, pre-amplifier, and constant fraction discriminator, and its output was processed by time-correlated single photon counting. The instrument response function, measured with a LUDOX (DuPont) suspension, had a full width at half-maximum of 245 ps. Decay curves were analyzed with decay analysis software supplied by IBH. Because the decay of all samples was double exponential, number-averaged, and intensity-averaged (31), lifetimes were calculated according to Equations 1 and 2.

$$\tau_{num} = \sum \alpha_i \tau_i \Big/ \sum \alpha_i \qquad \text{(Eq. 1)}$$

$$\tau_{int} = \sum \alpha_i \tau_i^2 \Big/ \sum \alpha_i \tau_i \qquad \text{(Eq. 2)}$$

RESULTS

The Most Prominent Fraction of the cHMGA in Chironomus Cells Is Phosphorylated by CK2 at Two Sites within Its Acidic Tail—Previously we reported that cHMGA is phosphorylated at distinct sites by Cdc2, MAP kinase, and PKC, respectively (19). These conclusions were drawn from peptide mapping studies of *in vivo* ^{32}P-labeled protein. Because the major fraction of the protein, having the highest electrophoretic mobility, was not detectable on autoradiograms, and a phosphatase treatment of this form did not change its mobility, we considered this form as dephosphorylated cHMGA. Unexpectedly, mass spectroscopic analysis of native cHMGA revealed that the major fraction of the protein had an M_r of 10,544. Because the calculated relative mass of the cHMGA protein is 10,340, this value suggested that this protein fraction could be doubly phosphorylated and singly acetylated (Fig. 1, *A* and *B*, Table I). Minor fractions with M_r of 10,383 and 10,463 could reflect protein forms that carry a single acetylation and both one acetylation and one phosphorylation, respectively (Table I). The fraction of M_r of 10,623 could reflect the protein with three phosphorylations and a single acetylation (Table I, Fig. 1*B*). To map the sites of modifications we isolated the main fraction of native cHMGA protein and digested the protein with trypsin. Peptide separation followed by mass analysis showed that the main subfraction of the C-terminal peptide 85–98 was doubly phosphorylated and that the N-terminal peptide 1–7 was acetylated (Table I, Fig. 1*D*). A small portion (<20%) of the peptide 85–98 was found in a singly phosphorylated form. Peptide 85–98 contains two serine residues in a sequence matching the consensus sequence phosphorylated by casein kinase 2; however, these two serine residues do not represent a preferred recognition site (32). To determine whether CK2 can modify Ser-96 and Ser-97 residues, we incubated the cHMGA recombinant protein in the presence of the CK2 and [γ-^{32}P]ATP. Rapid incorporation of labeled phosphate into the protein was observed (Fig. 1*C*). In contrast, under the same conditions the native protein was labeled only marginally (Fig. 1*C*, *inset*), indicating that nearly all CK2 target residues are already modified *in vivo*. To check whether the phosphorylated sites *in vitro* are identical to those modified *in vivo*, we digested cHMGA with trypsin and analyzed the products by HPLC. Two radioactive peptides were found (Fig. 1*E*, *rec. cHMGA/CK2 modified*). Their retention times were identical to those of the singly and doubly phosphorylated peptide 85–98 (Fig. 1*D*), indicating that Ser-96 and Ser-97 are phosphorylated by CK2 *in vivo*.

Taken together, our present and previous results (19) show that the cHMGA protein has at least five distinct phosphorylation sites located at serine residues at positions 3, 22, 72, 96, and 97 (Fig. 2). Phosphorylation of residues 96 and 97 appears to have a constitutive character, because more than 90% of the protein extracted from *Chironomus* cells is phosphorylated at both sites. The fully dephosphorylated form of the protein was rare, suggesting that in cells only minute amounts of this form are present. Up to 40% of the cHMGA population occurs in a form that is additionally phosphorylated at Ser-22 by MAP kinase (19). The fraction of protein phosphorylated at Ser-3 by Cdc2 kinase is below 5% and reflects the portion of cells that are in the late G_2 and M phases. Furthermore, a small fraction, usually below 2% of the cHMGA protein, was found to be modified at Ser-72. This amino acid represents a putative target site of PKC (19).

TABLE II
Spectral properties of cHMGA mutants and β-mercaptoethanol modified with acrylodan

Acrylodan adduct with	Emission maximum	Quantum yield ϕ[a]
	nm	
In buffer[b]		
β-Mercaptoethanol	538	0.18
(Cys-3)cHMGA	535	0.27
(Cys-3)cHMGA[P^{CK2}]	534	0.18
(Cys-15)cHMGA	532	0.41
(Cys-15)cHMGA[P^{CK2}]	535	0.47
(Cys-94)cHMGA	519	0.23
(Cys-94)cHMGA[P^{CK2}]	531	0.48
In 6 M guanidine·HCl + buffer[b]		
β-Mercaptoethanol	541	0.34
(Cys-3)cHMGA	539	0.43
(Cys-3)cHMGA[P^{CK2}]	539	0.44
(Cys-15)cHMGA	548	0.61
(Cys-15)cHMGA[P^{CK2}]	549	0.57
(Cys-94)cHMGA	523	0.28
(Cys-94)cHMGA[P^{CK2}]	540	0.51

[a] Quantum yield determinations were made versus acrylodan-β-mercaptoethanol ($\phi = 0.18$) according to Ref. 33.
[b] Buffer composition: 10 mM MOPS/NaOH, 100 mM sodium acetate, 100 μg/ml bovine serum albumin, pH 7.2.

TABLE III
Summary of the lifetime measurements

Sample	τ_1[a]	τ_2	α_1	α_2	τ_{num}	τ_{int}	τ_{num}/ϕ
	ns	*ns*			*ns*	*ns*	
(Cys-3)cHMGA	0.56	2.18	0.74	0.26	0.98	1.48	3.63
(Cys-3)cHMGA[P^{CK2}]	0.58	2.24	0.73	0.27	1.03	1.54	5.72
(Cys-15)cHMGA	0.65	2.34	0.68	0.32	1.19	1.70	2.90
(Cys-15)cHMGA[P^{CK2}]	0.63	2.26	0.68	0.32	1.15	1.64	2.45
(Cys-94)cHMGA	0.68	3.15	0.63	0.37	1.59	2.46	6.91
(Cys-94)cHMGA[P^{CK2}]	0.74	2.75	0.55	0.45	1.64	1.64	3.42

[a] τ_1 and τ_2 and α_1 and α_2 are lifetimes and pre-exponential factors, respectively, for the two decay components. The values of τ_{num} and τ_{int} were calculated according to Equations 1 and 2.

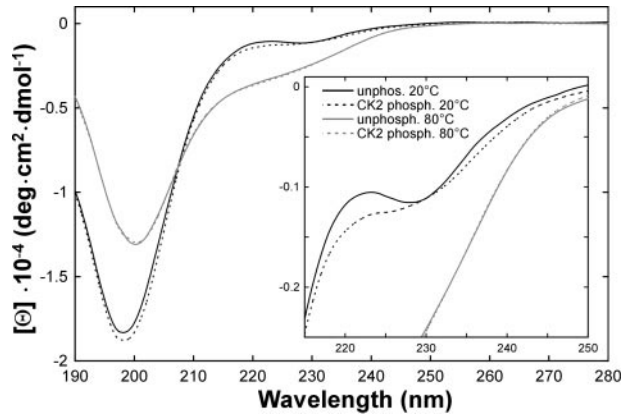

FIG. 4. **CD spectra confirm conformational changes of cHMGA induced upon CK2 phosphorylation.** The spectra of (Cys-94)cHMGA (*dashed lines*) and (Cys-94)cHMGA[P^{CK2}] (*solid lines*) were recorded in 10 mM sodium phosphate, pH 7.5 at 20 °C (*black lines*) or 80 °C (*gray lines*) in the range of 190–280 nm. *Inset*, enlarged representation of CK2 modification-induced alteration in the range of 215–250 nm. *deg*, degree.

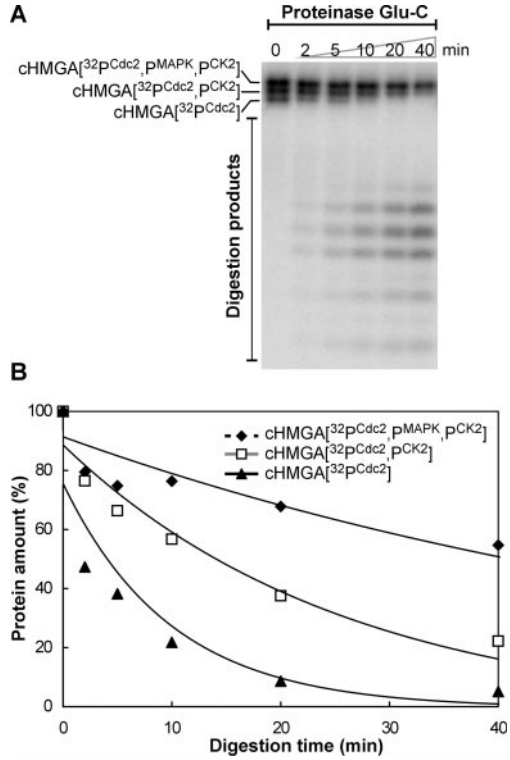

FIG. 5. **Time course of digestion of phosphoforms of the cHMGA protein.** The recombinant, native, and native Ser-22-phosphorylated proteins were phosphorylated by Cdc2 kinase in the presence of [γ-^{32}P]ATP at the residue Ser-3, yielding cHMGA[$^{32}P^{Cdc2}$], cHMGA[$^{32}P^{Cdc2}$,P^{CK2}], and cHMGA[$^{32}P^{Cdc2}$,P^{MAPK},P^{CK2}] forms. The three phosphoforms were mixed in the molar ratio of 1:1:1 and digested by proteinase Glu-C. At the indicated times, the reaction was terminated by addition of CCl_3COOH to a final concentration of 33%. The precipitated mixture of proteins and peptides was separated on 15% acrylamide gels in the discontinuous acetic acid/urea/Triton system (*A*). The gel was dried, and the radioactivity in the individual protein bands was quantified by phosphorimaging. *B*, analysis of the data from *A*. The ratio of [intact protein]$_t$ to [intact protein]$_0$ was plotted against time. The lines are theoretical curves calculated from the relationship [intact protein]$_t$/[intact protein]$_0$ = e^{-kt}. The calculated half-life times of cHMGA[$^{32}P^{Cdc2}$], cHMGA[$^{32}P^{Cdc2}$,P^{CK2}], and cHMGA[$^{32}P^{Cdc2}$,P^{MAPK},P^{CK2}] were 8.9, 19.9, and 53.2 min, respectively.

The consequences of the HMGA phosphorylation on protein binding to DNA have been analyzed (12, 14, 17–19, 22, 23). Those studies led to the conclusion that this type of posttranslational modification attenuates the strength of protein-DNA binding. However, it is unknown how changes in the binding affinity are accomplished and how phosphorylation of distinct sites affects the conformation of HMGA proteins. To obtain insights into these processes we examined the changes in protein structure and binding properties by means of hydroxyl radical protein footprinting, limited proteolytic digestion, and fluorescence and CD spectroscopy.

Fluorescence and CD Spectroscopy Reveals CK2-induced Structure Alteration—Single cysteine mutants of cHMGA labeled covalently with acrylodan were used to study the conformational changes of the protein in response to CK2 phosphorylation (Fig. 3, *A–C*). The fluorescence intensity and/or the position of the fluorescence emission maximum of this label depend on its microenvironment (33), and therefore its fluorescence can be used as a sensitive indicator of conformational changes in proteins (34–36). For this purpose, we constructed three mutants of cHMGA in which Ser-3, Ala-15, or Val-94 was substituted with a cysteine residue. After production in bacteria and purification, a portion of each mutant was phosphorylated *in vitro* by CK2. Both the phosphorylated and non-phosphorylated single cysteine mutants were then modified with acrylodan and purified.

The spectral properties of the acrylodan attached to cHMGA were different, depending upon the location of the label within the polypeptide chain, and were differentially affected by phosphorylation (Fig. 3). They are distinct in their quantum yields, their maxima of fluorescence emission (Fig. 3; Table II), and their lifetimes (Table III), suggesting that the fluorophore at each position is in a distinct environment. The phosphorylation of the protein by CK2 at Ser-96 and Ser-97 results in strong red shifts of the emission spectra of acrylodan at positions 15 and 94. In this case the phosphorylation seems to create a more polar environment for the probes (Fig. 3, *B* and *C*; Table II). For the protein labeled with acrylodan at Cys-3 phosphorylation by CK2 led to a weak blue shift, which seems to indicate a decrease in the polarity of the environment around the probe (Fig. 3*A*; Table II). The phosphorylation-dependent changes in the spectral properties of the proteins labeled at positions 3 and 15 disappeared in the presence of 6 M guanidine·HCl, suggesting structural differences between phosphorylated and non-phosphorylated forms of cHMGA (Fig. 3, *D* and *E*). In contrast, for the protein labeled at position 94 the great differences in fluo-

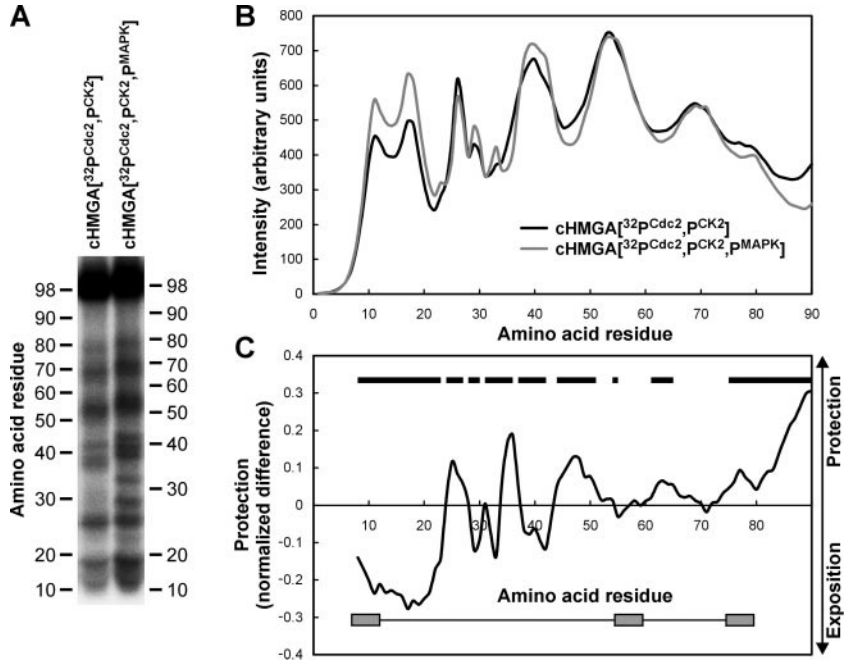

FIG. 6. **Phosphorylation at Ser-22 affects the conformation of the cHMGA protein.** *A*, representative electrophoretic patterns of hydroxyl radical digestions of the cHMGA[$^{32}P^{Cdc2}$] and cHMGA[$^{32}P^{Cdc2}$,P^{MAPK}] proteins from an individual experiment. *B*, plot of corrected PhosphorImager intensities. *C*, difference plot showing averaged data from eight independent experiments. Positive values mean less cutting in cHMGA[$^{32}P^{Cdc2}$,P^{MAPK}] compared with cHMGA[$^{32}P^{Cdc2}$] at this particular position. *Bold lines above the plot* indicate regions where the observed MAPK-induced protection or exposition was statistically significant according to a Student's *t* test (significance level of $\alpha = 0.05$). The schematic primary structure of the cHMGA protein with AT-hooks (*boxes*) is shown in the *lower part* of the panel.

rescence intensity between phospho and dephosphoforms persists even in the presence of 6 M guanidine·HCl (Fig. 3, *C* and *F*). This effect can be attributed to the vicinity of the fluorophore and the site of phosphorylation on the primary structure level. Because free acrylodan coupled to β-mercaptoethanol has an emission maximum of 538 nm (Table II), the emission maximum of 523 nm of the label at position 94 in the non-phosphorylated protein suggests that, even in the presence of 6 M guanidine·HCl, this region of the protein is not fully unfolded.

Analysis of fluorescence decays of acrylodan-labeled samples revealed that for all samples the decays were double exponential. The lifetimes of the two decay components were quite similar for all samples and were not affected greatly by phosphorylation (Table III). The observation that the lifetimes of acrylodan were relatively insensitive to phosphorylation and that the quantum yields were changed as a function of phosphorylation suggests that the quantum yield changes were entirely due to differences in static quenching. This can be illustrated by calculating the ratio of τ_{num} to quantum yield (Table III). In the absence of static quenching τ_{num} and quantum yield should be proportional (37). The data in Table III show the existence of large differences in this ratio between different proteins and between phosphorylated and unphosphorylated forms of the same protein. Thus, it can be concluded that acrylodan fluorescence detected conformational changes of the protein in response to phosphorylation.

Iodide is a collisional quencher, whose efficiency depends on the polarity of the environment of the fluorophore. For each of the analyzed forms of the cHMGA protein a strong quenching was observed (Fig. 3, *G–I*). Because a straightening of the plots in the presence of denaturant was observed (Fig. 3*I*), the downward curvature of the Stern-Volmer plots must originate from multiple conformers of the protein rather than from multiple chemically distinct forms of the probe (38). Because the intensity-averaged lifetimes (Table III) did not change significantly in response to phosphorylation, any changes in quenching efficiency in response to phosphorylation could be interpreted as being due to changes in the accessibility to the quencher. The phosphorylation of the protein mutants resulted in a weakening of quenching. In the case of the Cys-94 mutant, the changes can be directly attributed to the vicinity of the phosphate groups. In contrast, changes of the quenching efficiency at positions 3 and 15 must reflect changes in protein conformation.

To confirm that the changes in fluorescence properties induced by CK2 phosphorylation indeed originate from perturbations in protein conformation, we analyzed the CD spectral properties of the phosphorylated and non-phosphorylated forms of cHMGA. The comparison of the CD spectra at 20 °C revealed small but distinct differences between the CK2-phosphorylated and unmodified proteins (Fig. 4, *black lines*). Phosphorylation by CK2 led to a slightly reduced molar ellipticity in the region around 200 nm and, perhaps more importantly, caused a local maximum at 223 nm and a local minimum at 228 nm, accompanied by a general reduction of the ellipticity in the region of 215–250 nm (Fig. 4, *inset*). Because the shapes of the CD spectra were different, the observed changes cannot be a result of limited accuracy in determination of protein concentration. To show that the alterations induced by CK2 phosphorylation were due to structural changes, we also measured the CD spectra of phosphorylated and non-phosphorylated forms at 80 °C, assuming the denaturation of the proteins at this temperature (Fig. 4, *gray lines*). At 80 °C the ellipticity at 208 nm was reduced, whereas in the range of 208 to ~255 nm it was increased. No difference between CK2-phosphorylated and non-phosphorylated cHMGA was observed upon denaturation.

Partial Digestion Confirms a Conformational Change of the cHMGA Protein by Individual Phosphorylations—Limited proteolytic digestion is a sensitive tool for the detection of the changes in the structure of proteins. The mixture of ^{32}P-labeled cHMGA[P^{Cdc2}], cHMGA[P^{Cdc2},P^{CK2}], and cHMGA[P^{Cdc2},P^{MAPK},P^{CK2}] was partially digested by proteinase Glu-C (Fig. 5). The experiment revealed differences between the various phosphoforms in the susceptibility for degradation. Quantification of the radioactive bands of the labeled proteins showed that the resistance of the protein to degradation increased with the progress of the phosphorylation. Fitting of the experimental points to the single exponential decay function enabled calculation of the half-life for the individual protein forms (Fig. 5*B*). The half-life value of 53 min for cHMGA[P^{Cdc2},P^{MAPK},P^{CK2}] was 2.7 and 6 times greater than the values obtained for cHMGA[P^{Cdc2},P^{CK2}] and cHMGA[P^{Cdc2}], respectively. Because the sites that can be attacked by this protease are not in the proximity of the

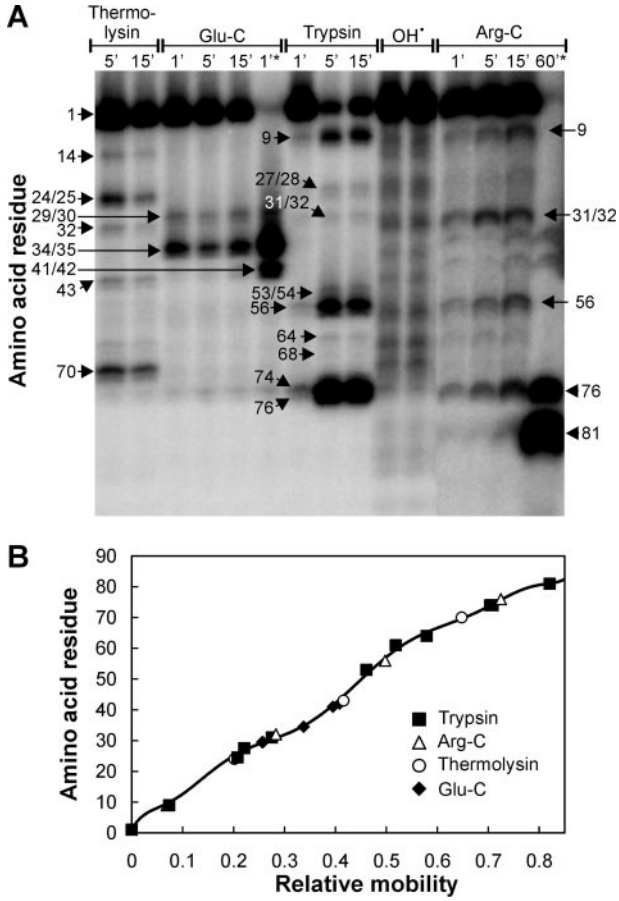

FIG. 7. **Molecular weight markers and assignment of the bands for protein footprinting using C-terminal labeling at Ser-96 and Ser-97 residues.** *A*, molecular weight markers were generated by site-specific cleavage of ^{32}P end-labeled cHMGA [P^{CK2}] with thermolysin, endoproteinases Glu-C (5 ng) and Arg-C (20 ng), and trypsin for the indicated time. Lanes marked with an *asterisk* represent extended digestions with 50 ng of Glu-C or 0.5 μg of Arg-C. *OH*•, hydroxyl radical lanes show peptide patterns of the protein digested with the chemical proteinase. *B*, plot of size of peptide markers *versus* relative mobility. The relative mobility of intact cHMGA was defined as 0, and the most rapidly migrating band of hydroxyl radical cleavage was defined as 1.

phosphorylation sites, the observed differences in the susceptibility to proteolysis suggest different conformations of the phosphoforms. A similar experiment in which cHMGA[P^{CK2}] and cHMGA[P^{MAPK},P^{CK2}] were compared revealed that phosphorylation at Ser-22 by MAPK increases resistance of the protein to digestion (data not shown). From these experiments the conclusion can be drawn that an increase in the extent of cHMGA phosphorylation is accompanied by an augmentation of structural compactness and metabolic inertness of the protein. Interestingly, phosphorylation within the C termini of structurally unrelated HMG1 box (HMGB) proteins has a similar effect on protein conformation, as manifested in increased protein stability (39). This may suggest that phosphorylation of HMG proteins by CK2 and MAPK may play an important role in regulating the turnover rates of these proteins.

Protein footprinting is a technique that can monitor structural changes in proteins (40). Using phosphorylation at Ser-3 for end labeling of the protein (10), we compared the native protein, modified *in vivo* at Ser-22 by MAP kinase, with the unphosphorylated protein. The resulting digestion patterns easily showed visible differences between the proteins (Fig. 6*A*). Quantitative analysis according to Frank *et al.* (10) enabled localization of the changes in accessibility induced by MAPK phosphorylation (Fig. 6*B*). For clearer documentation we transformed the data in plots of normalized differences (Fig. 6*C*). The region of residues 9–23 was exposed by modification of Ser-22, whereas small regions around residues 25–27, 34–37, and 44–51 and the whole C terminus from residue 76 were protected. Thus, among the three putative DNA-binding sites of the protein, only the solvent accessibility of the first AT-hook appears to be directly affected by MAPK phosphorylation.

In another set of experiments we investigated the modification of Ser-3, which is the target for Cdc2 kinase. To obtain more detailed information on the effect of this modification to the conformation of the protein and its DNA binding, we labeled the C terminus of cHMGA with ^{32}P using CK2 and performed hydroxyl radical footprinting analysis. For the size assignment of the bands in the hydroxyl radical patterns, a series of limited digestions with the sequence-specific proteinases thermolysin, trypsin, proteinase Glu-C, or proteinase Arg-C were performed to generate end-labeled peptides of defined length (Fig. 7, *A* and *B*). The comparison of the peptide patterns obtained by digestion of the CK2-labeled protein in its Cdc2-phosphorylated and non-phosphorylated forms revealed only slight differences. There were only a few regions for which the susceptibility was significantly altered (Fig. 8), with the strongest effect in the region comprising the second AT-hook (residues 52–58).

Phosphorylation of cHMGA by CK2 and Cdc2 Affects DNA Binding of the AT-hooks in the Proximity of the Phosphorylation Site—Because the primary function of HMGA proteins is binding to DNA, we analyzed how the individual phosphorylations that appear to occur *in vivo* affect the organization of the cHMGA-DNA complexes. Poly(dA-dT)·poly(dA-dT) and a 34-base pair fragment of the IFNβ promoter were footprinted on the end-labeled proteins. For the analysis of the effects of the phosphorylation at Ser-96/97 and Ser-22 the protein was labeled at Ser-3, whereas labeling at Ser-96/97 using CK2 was used to study changes in the complex organization upon phosphorylation by Cdc2 kinase at Ser-3.

Using the Ser-96/97 labeling we found that all three AT-hooks are involved in binding to poly(dA-dT)·poly(dA-dT) (Fig. 9*A*). In contrast, binding to the IFNβ fragment involved mainly the regions around the second and third AT-hooks (Fig. 9*B*). These results are in good agreement with previous studies in which the Ser-3 labeling was used (10).

Simultaneous phosphorylation of the protein by Cdc2 and CK2 resulted in significant changes in the protection patterns. In both complexes, with poly(dA-dT)·poly(dA-dT) and IFNβ, an impairing of the contacts via the third AT-hook was observed (Fig. 9, *C* and *D*). In the complex with IFNβ, an increase of the extent of protection in the regions flanking the central AT-hook was concomitant with an attenuation of protection in the region comprising the third AT-hook (Fig. 9*D*). Consecutive modification of the protein at Ser-22 by MAP kinase did not significantly change the protection pattern in protein footprints obtained with either poly(dA-dT)·poly(dA-dT) or IFNβ (Fig. 9, *C* and *D*, *gray lines*). Thus, it appears that this phosphorylation has no direct effect on cHMGA binding to the studied ligands.

DISCUSSION

Extensive phosphorylation is a characteristic feature of the mammalian (41, 42) and invertebrate (19, 43) HMGA proteins. Different kinases have been shown to be involved in phosphorylation of this group of proteins. In our previous study, we have reported that up to 40% of the cHMGA protein in *Chironomus* cells is phosphorylated by Cdc2 kinase, MAPK, and PKC (19). In these studies the phosphorylation sites were mapped in the protein that was *in vivo* pulse-labeled with radioactive orthophosphate. This technique leads to the detec-

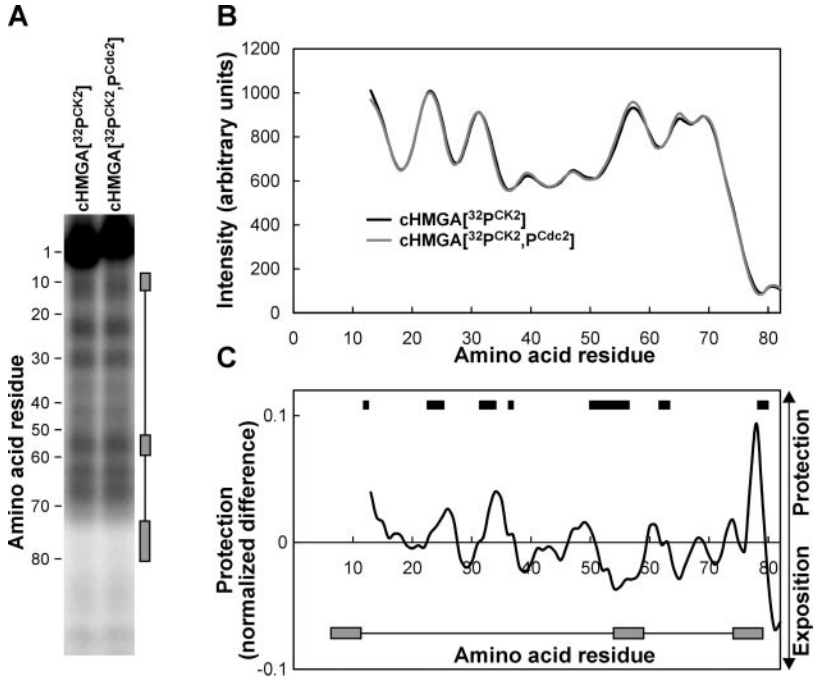

FIG. 8. **Changes in the conformation of the cHMGA protein upon phosphorylation by Cdc2 at Ser-3.** *A*, representative electrophoretic patterns of hydroxyl radical digestions of the cHMGA[$^{32}P^{CK2}$] and cHMGA[$^{32}P^{CK2}$,P^{Cdc2}] proteins from an individual experiment. *B*, plot of corrected PhosphorImager intensities. *C*, difference plot showing averaged data from 12 independent experiments. Positive values mean less cutting in cHMGA[$^{32}P^{CK2}$,P^{Cdc2}] compared with cHMGA[$^{32}P^{CK2}$] 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 (significance level of $\alpha = 0.05$). The schematic primary structure of the cHMGA protein with AT-hooks (*boxes*) is shown in the *lower part* of the panel.

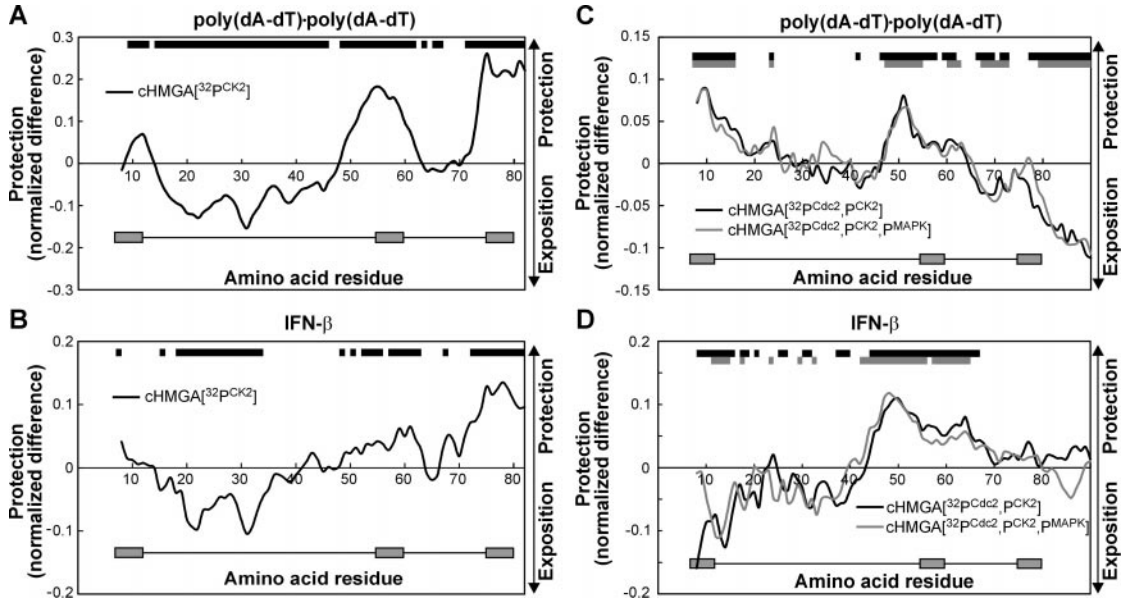

FIG. 9. **Protein footprints of poly(dA-dT)·poly(dA-dT) (*A* and *C*) and IFNβ fragment (*B* and *D*) on different phosphoforms of the N- (*A* and *B*) and C-terminally (*C* and *D*) labeled cHMGA.** Difference plots showing averaged data from 6–8 independent experiments. Positive values mean less cutting in DNA-bound cHMGA 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 (significance level of $\alpha = 0.05$). The schematic primary structure of the cHMGA protein with AT-hooks (*boxes*) is shown in each panel.

tion mainly of those phosphorylation sites that are frequently modified during labeling time. Because cHMGA protein has a long turnover rate (44), we were unable to detect the sites of phosphorylation at Ser-96 and 97 by radioactive labeling (19) because phosphorylation at these sites seems to be coupled to the rate of protein synthesis. The presented analysis, based on mass spectroscopic measurements, revealed that nearly the entire population of cHMGA is phosphorylated by CK2 at Ser-96 and Ser-97. Our previous and present results suggest that phosphorylation by CK2 is coupled to translation, whereas the other kinases confer specific properties to the protein in a dynamic and reversible manner. Because the modification by CK2 affects nearly the entire population of the protein in the cell and appears to persist through longer periods, we called this constitutive phosphorylation (39). In contrast, the other phosphorylations within the cHMGA were termed facultative (39).

Studies performed in several laboratories have shown that the DNA binding affinity of HMGA proteins is attenuated upon phosphorylation (12, 14, 17–19, 22, 23). Our previous analyses of the binding properties of the insect HMGA revealed that coincident phosphorylation of the protein at two positions is necessary to elicit a significant change in the affinity of the protein to AT-rich satellite DNA (19). In this work we investigated the consequences of phosphorylation of cHMGA with respect to protein conformation and the organization of the cHMGA-DNA complex.

HMGA proteins are usually considered randomly coiled polypeptides. Indeed, circular dichroism spectra of mammalian HMGA1a protein suggest that about 70% of the polypeptide

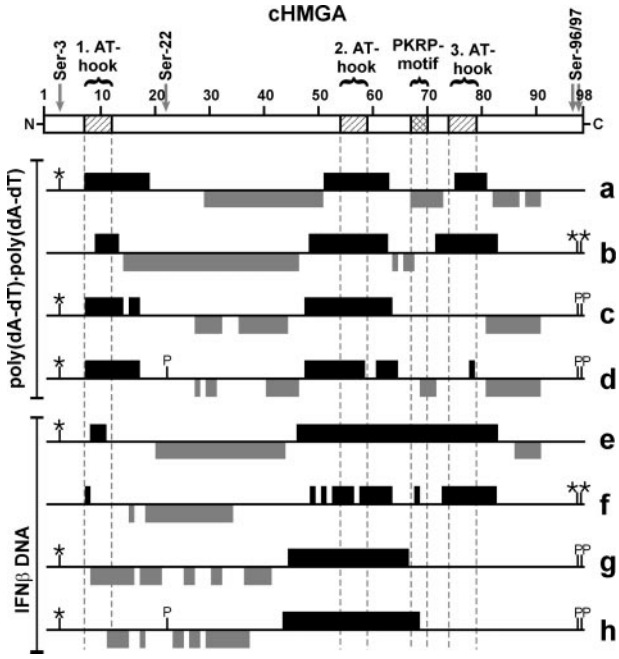

FIG. 10. **Summary of the footprinting experiments.** The *upper box* schematically represents the cHMGA protein. The *hatched* and *checkered areas* indicate the positions of the DBDs and the PKRP sequence motifs, respectively. Regions of cHMGA that are significantly protected from, and exposed to, the hydroxyl radical cleavage in the presence of DNA are indicated by *horizontal black* and *gray boxes*, respectively. *P* indicates a phosphate group; *asterisks* indicate sites of end labeling with ^{32}P. Data in *a* and *e* are from (10).

does not adopt any defined secondary structure (45). When we fitted the CD spectra of the cHMGA with secondary structure estimation software using the algorithm of Yang *et al*. (46), the results indicated that as much as 23% of the polypeptide may be in the β-sheet conformation and that about 21% may be in β-turns. Thermal denaturation of the protein changed the CD spectra in such a way as to emphasize the presence of conformational preferences of the native protein. However, the estimation of the secondary structure of the denatured protein yields a higher content of β-sheet ($\sim$48% β-sheet and $\sim$6% β-turn), so that such estimations should be interpreted cautiously, especially in the case of HMGA proteins. The highly reproducible digestion patterns obtained using either hydroxyl radicals or proteinases suggest that HMGA1a, HMGA1b, HMGA2, and cHMGA have distinct conformations (10, 12, 14). The results presented in this study strongly indicate that the polypeptide of the cHMGA is not fully randomly coiled either and that phosphorylation by CK2, MAPK, and, to a lesser extent, Cdc-2 modulate its spatial arrangement.

The protein footprinting DNA binding studies show that phosphorylation at two sites must occur to impair contacts between a single AT-hook and DNA. The observed effects of protein phosphorylation are distinct with respect to the type of DNA bound to the protein (10). Phosphorylation of both termini of the protein leads to a reduction of the contacts of the third AT-hook in the complex with poly(dA-dT)·poly(dA-dT) (Fig. 10, *a* and *c*). In the case of IFNβ fragment binding this double phosphorylation causes a loss of the interaction of the first and third AT-hooks (Fig. 10, *e* and *g*). These findings resemble the results obtained during the studies of phosphorylation of human HMGA1b (14) and murine HMGA2 (12). For those proteins, simultaneous phosphorylation by CK2 and Cdc2 leads to changes in the nature of the contacts between individual elements of IFNβ and a single AT-hook. In HMGA1a, this phosphorylation affects binding of the N-terminal AT-hook to the positive regulatory domain III-1 element. In contrast, post-translational modification of the HMGA2 results in structural perturbations of the binding of the central and C-terminal AT-hook to positive regulatory domain II and negative regulatory domain I elements, respectively.

Consecutive phosphorylation of the Ser-22 by MAPK has no additional effect on the extent of contacts between the protein and either poly(dA-dT)·poly(dA-dT) or the IFNβ fragment (Fig. 10, *d* and *h*). Modification of this residue may be important for modulation of the cHMGA interaction with other types of DNAs or proteins.

The biological significance of multiple sites of phosphorylation in the HMGA proteins is currently unknown. In addition to modulating DNA binding, the phosphorylation of specific residues may affect the turnover rate and cellular distribution of the proteins. Dephosphorylation of the C-terminal serines in the insect HMGA protein leads to conformational changes that, under *in vitro* conditions, facilitate degradation of the protein. Because the phosphorylation of the C-terminal acidic "tails" appears to be common within the HMG families (39), it is possible that it represents a widespread mechanism to increase stability.

Cytological studies have revealed that HMGA proteins occur mainly in the G/Q and C bands of mitotic chromosomes (47), which represent constitutive heterochromatin. In interphase nuclei of mammalian cells (48, 49) and in the *Drosophila* polytene chromosomes, HMGA and the related D1 protein (50), respectively, are also abundant in heterochromatin. But the HMGA proteins have also been localized to decondensed chromatin, to chromosomal puffs, and to nucleolus organizer (44, 48), which are chromosomal sites active in transcriptional processes. Thus, the presence of subpopulations of HMGA proteins may suggest their functional variability, which could be achieved by distinct phosphorylations.

The HMG1 box domain proteins (HMGB) and the nucleosome-binding HMGN proteins are structurally unrelated to HMGA but share with HMGA several physicochemical properties, *e.g.* their small size, amphipathic character, and unusual solubility in acids. Therefore, phosphorylation by particular kinases can have common consequences for proteins that are members of the HMG family. Microinjection experiments of phosphorylated and non-phosphorylated HMGB proteins (51), as well as mass spectroscopic studies on the subcellular distribution of HMGN proteins (52), suggest that the dephosphorylated and phosphorylated states are characteristic of nuclear and cytoplasmic locations. Future studies could answer this question by comparing the modification status within the HMGA proteins in the nuclear and cytoplasmic fractions.

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GENES: STRUCTURE AND REGULATION:

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