Conformational Changes of DNA Induced by Binding of *Chironomus* High Mobility Group Protein 1a (cHMG1a)

**REGIONS FLANKING AN HMG1 BOX DOMAIN DO NOT INFLUENCE THE BEND ANGLE OF THE DNA**

Ewa Heyduk‡, Tomasz Heyduk‡§, Peter Claus§, and Jacek R. Wiśniewski¶

From the ‡Edward A. Doisy Department of Biochemistry and Molecular Biology, St. Louis University Medical School, St. Louis, Missouri 63104 and ¶ Zoologisches Institut-Entwicklungbiologie, Universität Göttingen, Humboldtallee 34A, D-37073 Göttingen, Germany

High mobility group (HMG) proteins are thought to facilitate assembly of higher order chromatin structure through modulation of DNA conformation. In this work we investigated the bending of a 30-base pair DNA fragment induced by *Chironomus* HMG1 (cHMG1a), and HMGI (cHMGI) proteins. The DNA bending was measured in solution by monitoring the end-to-end distance between fluorescence probes attached to opposite ends of the DNA fragment. The distance was measured by fluorescence energy transfer using a novel europium chelate as a fluorescence donor. These measurements revealed that the end-to-end distance in the 30-base pair DNA was decreased from ~100 Å in free DNA to ~50.5 Å in cHMG1a-DNA complex. The most probable DNA bending angle consistent with these distance measurements is about 150°. The deletion of the charged regulatory domains located close to the C terminus of the HMG1 box domain of cHMG1a protein had no effect on the induced bend angle. The ability to induce a large DNA bend distinguishes the cHMGI from the cHMG1 protein. Only small perturbation of the DNA conformation was observed upon binding of the cHMGI protein. A strong DNA bending activity of cHMG1a and its relative abundance in the cell suggests that this protein plays a very important role in modulation of chromatin structure.

Local modulation of DNA structure is an important event in the organization of chromatin. The TATA box-binding regulatory protein induces large conformational changes in DNA, in particular helix unwinding, widening of the minor groove and DNA bending (1). Proteins carrying conserved HMG1 box domain (HMG1-BD) and the (K/R)XRGRP (AT-hook) motif have been found to modulate the DNA conformation by interacting primarily with the minor groove of DNA (for a review, see Ref. 2).

The ~80-amino acid HMG1-BD DNA-binding element is found in eukaryotic proteins involved in diverse physiological processes, including regulation of transcription and development (3). Each of the HMG1-BDs falls in one of the two classes: those that do or do not recognize specific DNA motifs. The HMG1-BDs of sex-determining region Y factor (SRY) and lymphoid enhancer-binding factor (LEF-1) are the best understood members of the first class. The highly abundant HMG1/2-type proteins and the upstream binding factor are examples of the second class. A common feature of all HMG1-BDs is their ability to distort the conformation of B-DNA. The induced DNA bend by HMG1-BD-carrying proteins is thought to facilitate organization of higher order chromosomal structure (for reviews, see Refs. 2 and 3).

The angles of the DNA bend induced by binding of HMG1-BDs of SRY and LEF-1 proteins have been determined by the circular permutation assay (4–6) and NMR spectroscopy (7, 8). The NMR studies revealed that the HMG1-BDs of human SRY and mouse LEF-1 bend their target DNAs by ~70–80° and ~107–127°, respectively. The bending of DNA induced by binding of sequence nonspecific HMG1-BD has been only indirectly demonstrated, using the ligase-mediated circularization assay (9–13).

The sequences flanking the HMG1-BD at its C terminus are able to modulate its affinity for DNA. Changes in the number of positive and negative charges within the C-terminal portion of the insect HMG1 protein (14) increase and decrease the DNA-binding affinity of the protein, respectively (15). Similarly, a C-terminal addition of mainly positively charged residues to the insect HMG1 protein (14) increase and decrease the DNA-binding affinity. The presence of this extension has also been suggested to be essential for DNA bending by HMG1-BD of LEF-1 (6).

An AT-hook DNA-binding motif is the characteristic feature of the proteins of the HMG1/Y family, which comprises four structurally related proteins: the mammalian proteins HMGI and HMGY (17, 18), HMGI-C (19), and an insect cHMG1 (20). Proteins containing several AT-hook motifs have been found in plants (for a review, see Ref. 21) and *Drosophila* (D1 protein; Ref. 22). Binding of HMGI to a DNA fragment of the positive regulatory domain II of the β-interferon gene resulted in a reduction of an intrinsic curvature of a fragment by 13° (23).

In this report we analyzed the conformational changes of a 30-bp DNA upon binding of the *Chironomus* HMG1a and HMGI proteins. Employing fluorescence resonance energy transfer (FRET) distance measurements, we found that the cHMG1a protein bent the 30-bp DNA fragment by about 150°. We also show that the changes in the DNA-binding affinity of cHMG1a produced by deletion of the charged sequences flank-
ing the HMG-BD have no apparent effect on the angle of DNA bending. The binding of the cHMG1 protein induced only a small perturbation of DNA conformation.

EXPERIMENTAL PROCEDURES

Materials—AMCA-NHS (7-amino-4-methylcoumarin-3-acetic acid, succinimidyl ester) was purchased from Boehringer Mannheim, AMCA(13)-S-S-Py (N-(6-7-amino-4-methylcoumarin-3-acetamido)-hexyl-3’-(2-pyridylthio)propionamide) was from Pierce, and CY5 (nonfunctional N-hydroxysuccinimide ester) was from Amersham Corp. Diethylaminomipentanedioic acid anhydride (DTPA anhydride) and EuCl3 were from Aldrich. All other chemicals were of highest purity commercially available. The thiol-reactive europium chelate, DTPA-AMCA(13)-S-S-Py, was prepared as described previously (24).

Construction of Expression Vectors—A polymerase chain reaction was used to introduce NdeI restriction site to the N-terminal end of the coding region of the cHMG1 cDNA clone (pCW126, Ref. 20). The polymerase chain reaction product was cloned into the vector pGEM-T (Promega), cut with NdeI/EcoRI and ligated with the NdeI-EcoRI fragment of pET3a to give the expression constructs. The cloned DNA inserts were sequenced to verify that only the intended changes had occurred. For the induction of expression, the constructs were transferred to Escherichia coli BL21(DE3). Bacteria carrying constructs for the expression of the cHMG1a protein and deletion mutant cHMG1a/102 were prepared as described previously (15).

Synthesis of cHMG1a, cHMGI, and Mutant Proteins in Bacteria—Transformed E. coli BL21(DE3) cells were grown at 37 °C in 5 ml of LB medium in the presence of 300 μg of ampicillin on a rotary shaker with 240 revolutions/min. A 12-h-old culture was used to inoculate 200 ml of fresh LB medium containing 20 mg of ampicillin. The culture was grown to an optical density of 0.4 measured at 600 nm. The expression of the HMG proteins was then induced by adding isopropyl-1-thio-β-D-galactopyranoside to a concentration of 1 mM. The induced culture was shaken for an additional 2 h under the same conditions. The cells were harvested in a centrifuge at 4 °C with 5000 × g. Cell pellets were frozen at −20 °C.

Extraction and Purification of HMG Proteins—Proteins were extracted from bacteria with 5% HClO4 by three thawing-freezing cycles. The supernatants were acidified with HCl to 0.5 M, precipitated with 6 volumes of acetone, and dried under vacuum. The extracted proteins were chromatographed on a MONO S column (Pharmacia, Uppsala, Sweden) using a NaCl gradient in 25 mM boric acid/NaOH, pH 9.4. The fractions containing recombinant proteins eluting as a major peak at about 0.4 M NaCl were pooled, desalted on a PD-10 column (Pharmacia), lyophilized, and finally, the HMG proteins were re-chromatographed on a Zorbax 300 SB C-18 reverse-phase column using an acetonitrile gradient in 0.1% trifluoroacetic acid/water.

The HMG1-BD of cHMG1a (cHMG1a/84) was prepared by limited digestion of the cHMG1a with trypsin as described previously (15).

Preparation of Fluorochrome-labeled DNA—All oligonucleotides were obtained from Midland Certified Reagent Co. (Midland, TX). The donor-labeled upper strand was prepared by using two approaches resulting in two different donor-labeled DNA molecules, differing in a size of the linker between DNA and the fluorochrome. In a first approach a reactive thiod group was introduced to a 5’ end of the oligonucleotide by a post-synthetic modification with cystamine of the 5’-phosphorylated oligonucleotide (25) and subsequent modification with thiol-reactive europium chelate (DTPA-AMCA(13)-S-S-Py; structure and properties described in Ref. 24). The product was purified by two successive Sephadex G-25 spin columns as described previously (24). This modification reaction produced a donor-labeled oligonucleotide with a 17 atom linker between the 5’-phosphorus atom and the coumarin ring of the europium chelate. To prepare a donor-labeled oligonucleotide with a shorter linker, a novel two-step procedure was used. The procedure described below allows europium chelate modification of an aliphatic amino group-containing oligonucleotides. In the first step, the 5’-aliphatic amino group was introduced by a post-synthetic modification with ethylendiamine of a 5’-phosphorylated oligonucleotide using procedures analogous to those used for cystamine modification (25). The 5’-amino oligonucleotide was then modified with AMCA-NHS by incubation with 10 μM oligonucleotide in 0.1 M sodium bicarbonate buffer (pH 8.3) for 4 h at room temperature with 2 mM AMCA-NHS. The excess of unreacted AMCA-NHS was removed on a Sephadex G-25 spin column, and the labeled oligonucleotide was further purified by high performance liquid chromatography using a 4.1 mm × 150-mm PRP-1 reverse phase column (Hamilton, Reno, NV). The column was run at 1 ml/min, and a linear gradient of 50 ml of 10–60% “B” was used. Buffer “A” was 25 mM triethylammonium acetate (pH 7.0), and buffer “B” was 25 mM triethylammonium acetate (pH 7.0) containing 50% acetonitrile. Fractions containing AMCA-labeled oligonucleotide were pooled and were dried in Speed-Vac (Savant Instruments Inc., Holbrook, NY). In the second step, 7-amino group of DNA-bound AMCA was modified with DTPA anhydride. Dry AMCA-labeled oligonucleotide was dissolved in 0.2 M HEPEs buffer (pH 7.7) and was reacted with DTPA anhydride (20 mg/ml) for 1 h at room temperature. The excess of unreacted DTPA was removed by running the sample twice through a Sephadex G-25 spin column. This two-step procedure results in donor-labeled DNA with a 6-atom linker between 5’ phosphorus atom and the coumarin ring of europium chelate. The structure of the europium chelate itself (Fig. 1B) is identical to the thiol-reactive europium chelates described previously (24).

CYS-labeled complementary oligonucleotide was prepared and purified using methods described previously (24).

To prepare fluorochrome-labeled DNA duplexes, equimolar amounts of europium chelate-labeled 30-nucleotide oligonucleotide were mixed with unlabeled complementary strand (donor-only 30-bp DNA) or with CYS-labeled complementary 30-nucleotide oligonucleotide (donor-acceptor 30-bp DNA) in 50 mM Tris buffer (pH 7.8), 100 mM NaCl containing 10 μM EDTA. The mixtures were heated to 95 °C for 30 s and were allowed to cool slowly to room temperature. Europium chloride (EuCl3) was added to hybridized DNA samples to a concentration of 5 μM, and after 30 min of incubation at room temperature, the excess of europium was removed on a Sephadex G-25 column. The labeled DNA was stored at −20 °C until needed.

Fluorescence Measurements—Steady-state fluorescence and polarization measurements were performed on a SLM 500C spectrophotometer (SLM Instruments, Inc., Urbana, IL) equipped with an L-format polarization accessory. Luminescence lifetime measurements were performed on a laboratory-built two-channel spectrophotometer with a pulsed nitrogen laser (LN300, Laser Photonics, Orlando, FL) as an excitation source (24). This instrument allows simultaneous acquisition of donor decay (at 620 nm) and sensitized acceptor decay curves (at 670 nm). The decay curves were analyzed according to Equation 1.

\[ I = \sum \gamma_{i} \exp(-t/\tau_{i}) \]  
(1)

\( I \) is the measured intensity at time \( t \) after the excitation pulse, and \( \alpha \) and \( \tau \) are the amplitude and the lifetime of the \( i \)th decay component, respectively. The decay curves were fitted to Equation 1 by a non-linear regression using SCIENTIST (Micromath Scientific Software, Salt Lake City, UT).

The energy transfer (\( E \)) between europium chelate and CY5 was calculated from measurements of luminescence lifetime of a donor in the absence (\( \tau_{d} \)) and in the presence of acceptor (\( \tau_{d}^{a} \)).

\[ E = 1 - \tau_{d}^{a}/\tau_{d} \]  
(2)

The distances between donor and acceptor were calculated according to Förster theory (26).

\[ R^{6} = R_{0}^{6}(1 - E/E) \]  
(3)

\( R \) is a distance between a donor and an acceptor, and \( R_{0} \) is a distance at which the energy transfer is 0.5. The \( R_{0} \) was calculated using standard procedures (28–30) and assumptions described by Selvin et al. (27, 31), according to Equation 4.

\[ R_{0} = 9.7 \times 10^{2} \left( \frac{J_{i}}{n_{i}^{2} \epsilon_{i}^{6}} \right)^{1/6} \]  
(4)

The orientation factor (\( \epsilon^{6} \)) was assumed to be 2/3 (24, 27, 31). The quantum yield of europium in europium chelates was assumed to be 100% D2O (27). The quantum yields of europium chelates in water and in water/D2O mixtures were calculated from Equation 5.

\[ q_{\text{Eu}} = \tau_{d}^{a}/\tau_{d} (100\% \text{ D}_{2}O) \]  
(5)

\( \tau_{d}^{a} \) is a lifetime in water or water/D2O mixture, and \( \tau_{d} \) is the lifetime in 100% D2O. All fluorescence experiments were performed in 10 mM Tris buffer (pH 7.8), 80 mM NaCl, 5% glycerol, 10 μM EDTA. In some experiments 1 mM MgCl2 was included, but the results were essentially identical to those in buffer with no magnesium.

RESULTS

Fluorochrome-labeled DNA—The overall design of the experiments was to use the changes in the end-to-end distance of a DNA fragment induced by binding of the cHMG proteins to...
Determine the extent of DNA deformation (bending). The end-to-end distance was measured in solution using FRET between fluorochrome probes attached to the opposite ends of a DNA fragment. Fig. 1 (A and B) shows a design of the fluorochrome-labeled 30-bp DNA molecules. The term donor-only DNA is used in this work to refer to a 30-bp DNA molecule with an europium chelate attached to the 5' end of the upper strand. The donor-acceptor DNA refers to a 30-bp DNA with an europium chelate attached to the 5' end of the upper strand and CY5 fluorochrome attached to the 5' end of the lower strand. We used europium chelates as donors in FRET measurements because these luminescence probes offer several important advantages compared with organic dye fluorochromes traditionally used for FRET experiments (24, 27, 30). The $R_0$ value of europium chelate-CY5 donor-acceptor pair calculated from absorbance and fluorescence emission spectra of the 30-bp fluorochrome-labeled DNA samples (data not shown) was 55 Å, a value identical to the one reported previously (24).

Both the cHMG1a and cHMGI proteins were able to form a complex with fluorochrome-labeled 30-bp DNA, as illustrated by DNA binding experiments in solution using polarization of fluorochrome-labeled DNA fluorescence as an indicator of protein-DNA complex formation (Fig. 1C). Fluorescence anisotropy values of DNA complexes with cHMG1a and cHMGI were different, although the molecular mass values of these proteins are similar (12.9 kDa (14) and 10.4 kDa (20), respectively), suggesting that the conformation of protein-DNA complex in both cases was different. The stoichiometry of cHMG1a-DNA complexes is not well established. In the presence of cHMG1a, there are probably two major species in solution (free DNA and a 1:1 protein-DNA complex) because: (i) protein concentrations used (0–1100 nM) for FRET measurements were not saturating (see Fig. 1C), (ii) the binding of cHMG1a to a short DNA fragment was shown to be non-cooperative (15), and (iii) cHMG1a was shown to preferentially bind to AT-rich sequences (14). Our 30-bp DNA fragment contains a 11-bp stretch of A and T nucleotides at its center, which should promote preferential binding of a single cHMG1a molecule to the center of the DNA fragment (binding site size for cHMG1a was shown to be 11–13 bp; Ref. 15).

The Effect of cHMG1a Binding on DNA Conformation—Fig. 2 shows the decay curves for donor-only 30-bp DNA in the absence and in the presence of cHMG1a. The decays were rigorously single-exponential in the absence and in the presence of cHMG1a, and the lifetimes of donor decay were not significantly changed in the presence of the protein. These data suggest that the donor molecules were not significantly perturbed by the binding of the protein to DNA. Such insensitivity of europium chelate decay to its microenvironment when attached to a macromolecule was observed previously (24). Single-exponential decays of donor-only samples allow straightforward analysis of the effects observed in donor-acceptor DNA.

Fig. 3 shows the decay curves of europium luminescence in donor-acceptor labeled 30-bp DNA in the absence of cHMG1a, with 250 nM cHMG1a, and with 1100 nM cHMG1a (Fig. 3, A–C, respectively). Without the protein, the donor decay was rigorously single-exponential and the determined lifetime was slightly smaller in comparison to donor-only sample (Table 1). With cHMG1a, the decay could no longer be fitted to a single-exponential function and a satisfactory fit could only be obtained using double-exponential function. The longer lifetime component decayed with a lifetime essentially identical to the one observed in the absence of the protein. The amplitude of this component decreased with an increase in protein concentration (Fig. 3, B and C). Therefore, we concluded that this component corresponded to a population of DNA molecules that were not bound to the protein. As already discussed, the concentrations of cHMG1a used in luminescence decay experiments were not saturating; thus, the presence of free DNA was expected. The shorter lifetime component (≈200 μs) appears only in the presence of cHMG1a with donor-acceptor DNA, and its amplitude increases with an increase of cHMG1a concentration. Thus, we concluded that this component corresponded to donor-acceptor 30-bp DNA complexed with cHMG1a. The smaller value of the lifetime of this component compared with a lifetime of donor-only DNA molecule suggests that the end-to-end distance of 30-bp DNA in complex with cHMG1a was drastically decreased compared with free DNA.

The assignment of the ≈200-μs component to a DNA in complex with cHMG1a was corroborated by two additional experiments. First, the decay of a sensitized acceptor emission in donor-acceptor DNA was measured in the presence and absence of cHMG1a (Fig. 4). In the case of the europium chelate-CY5 pair used in our studies, a lifetime of a donor is in the microsecond range and the lifetime of an acceptor is in the nanosecond range. Thus, any emission of the acceptor recorded in microsecond time scale would be originating from acceptors that were excited by FRET (24, 27, 31). Consequently, the lifetime of sensitized acceptor fluorescence should reflect the lifetime of the donor engaged in FRET. Without the protein (Fig. 3A), only a small background fluorescence is observed (the energy transfer in free DNA is very small, about 2%; see Table 1). Addition of the protein produced a large increase in the sensitized emission of the acceptor (Fig. 4, B and C) confirming...
parameters were as follows: a single-exponential function, illustrating that the data in these panels were not fitted satisfactorily by a single exponential function. The fitted could be observed only in sensitized emission of acceptor but protein. Its nature is unclear, since this short-lived component could be also observed in sensitized acceptor decays with the complex with cHMG1a. A very short-lived component (19766 samples), the lifetime shown is a component assigned to a protein-DNA complex. The results obtained with donor attached to DNA using a shorter linker (6-atom) are shown in this table. The table summarizes the results of distance measurements in 30-bp DNA fragment in the absence and in the presence of cHMG1a protein and its deletion mutants. In the case of multiexponential decays (donor-acceptor or double-exponential function (B and C). For comparison, the broken lines in panels B and C represent nonlinear fit of the decay data to a single-exponential function. The fitted parameters were as follows: A, $\tau_1 = 591 \mu s$ (amplitude = 100%); B, $\tau_1 = 544 \mu s$ (amplitude = 71%); $\tau_2 = 194 \mu s$ (amplitude = 29%); C, $\tau_1 = 585 \mu s$ (amplitude = 35%), $\tau_2 = 230 \mu s$ (amplitude = 65%).

The results obtained with donor attached to DNA using a shorter linker (6-atom) are shown in this table.

The NMR spectrum of cHMG1a and its deletion mutants is shown in Fig. 3. The table summarizes the results of distance measurements in 30-bp DNA fragment in the absence and in the presence of cHMG1a protein and its deletion mutants.

a The results obtained with donor attached to DNA using a shorter linker (6-atom) are shown in this table.

b In the case of multieponential decays (donor-acceptor + protein samples), the lifetime shown is a component assigned to a protein-DNA complex.

d a drastic increase of FRET in donor-acceptor 30-bp DNA with cHMG1a. The major component of sensitized acceptor emission decayed with a lifetime of $\sim 200 \mu s$, essentially identical to the lifetime of a short component of donor decay. These data confirmed the assignment of a $\sim 200-\mu s$ component to DNA in complex with cHMG1a. A very short-lived component ($\sim 20 \mu s$) could be also observed in sensitized acceptor decays with the protein. Its nature is unclear, since this short-lived component could be observed only in sensitized emission of acceptor but not in donor decay measured directly. Therefore, the $\sim 20-\mu s$ component is probably not related to FRET between donor and acceptor. In a second experiment, a possibility that the $\sim 200-\mu s$ component observed in the presence of cHMG1a could be due to the intermolecular energy transfer resulting from nonspecific aggregation of DNA with the protein was addressed. Decays of donor fluorescence were measured in an equimolar mixture of DNA duplexes labeled with donor only and with acceptor only, respectively (Fig. 5). We reasoned that if the $\sim 200-\mu s$ component was due to intermolecular energy transfer caused by cHMG1a-induced aggregation, efficient FRET should be observed also in this case where donor and acceptor were on separate DNA molecules. Fig. 5 shows that donor decays in this experiment remained single-exponential and unchanged in the presence of cHMG1a confirming that the $\sim 200-\mu s$ component reflects structural changes within the DNA molecule.

Table I summarizes the results of distance measurements in 30-bp DNA with and without cHMG1a. In its absence, the distance between the donor and acceptor was $\sim 100 \AA$ (the precision of this estimation is rather poor due to a small value of the energy transfer), which is compatible with a distance expected for 30-bp DNA duplex (see “Discussion”). In the protein-DNA complex, this end-to-end distance was decreased about 2 times to $\sim 50.5 \AA$, suggesting extensive DNA deformation induced by binding of cHMG1a.

The FRET measurements in the presence of cHMG1a were also performed using DNA labeled with europium chelate with

### Table I: Summary of energy transfer distance measurements in 30-bp DNA fragment in the absence and in the presence of cHMG1a protein and its deletion mutants

| Sample          | Lifetime$^a$ | Energy Transfer | Distance |
|-----------------|--------------|-----------------|----------|
| Donor           | 602.9 ± 2    |                 |          |
| Donor + cHMG1a | 616.7 ± 7    |                 |          |
| Donor-acceptor  | 586.4 ± 3    | 0.027 ± 0.01    | 100.0 ± 10 |
| Donor + cHMG1a | 560.0 ± 8    | 0.63 ± 0.04     | 50.3 ± 0.3 |
| Donor + cHMG1a/102 | 614.0 ± 1 | 0.62 ± 0.04     | 50.7 ± 0.3 |
| Donor-acceptor + cHMG1a/102 | 233.0 ± 23 | 0.60 ± 0.08     | 51.4 ± 3.0 |
| Donor + cHMG1a/84 | 606.0 ± 4 | 0.60 ± 0.08     | 51.4 ± 3.0 |
| Donor-acceptor + cHMG1a/84 | 243.9 ± 47 | 0.60 ± 0.08     | 51.4 ± 3.0 |

$^a$ The results obtained with donor attached to DNA using a shorter linker (6-atom) are shown in this table.

$^b$ In the case of multieponential decays (donor-acceptor + protein samples), the lifetime shown is a component assigned to a protein-DNA complex.
a longer linker (17 atoms; see “Experimental Procedures”). As expected, with a longer linker the energy transfer in a linear 30-bp DNA was decreased (to 0.015, corresponding to \( r = 110 \text{ Å} \)). Interestingly, the measured distance for protein-DNA complex (49.3 Å) was even shorter than the distance of 50.5 Å determined with the shorter linker.

To further confirm that the observed increase in FRET induced by cHMG1a was due to conformational changes in DNA, two additional control experiments were performed. In the first experiment, the order of the fluorescent donor/acceptor groups on 5' ends of 30-bp DNA was reversed. The FRET experiments with this DNA showed that the energy transfer for cHMG1a-DNA complex in this case was 0.68 \pm 0.03, corresponding to a distance of 48.5 \pm 1.2 Å, a value identical within the experimental error to the one observed previously. In the second experiment, a 30-bp DNA with a different sequence (CGGTCAGAAAATTATTTTAAATTTCCTTT) was used. As before, with cHMG1a a remarkable increase in FRET was observed for this DNA fragment. The energy transfer for protein-DNA complex was 0.71 \pm 0.04, corresponding to a distance of 47.4 \pm 1.5 Å, again the value very similar to the one observed previously.

FRET experiments with donor-acceptor 30-bp DNA were also performed with two deletion mutants of cHMG1a: cHMG1a/102 and cHMG1a/84. In cHMG1a/102 a C-terminal negatively charged domain of the protein was removed, and in cHMG1a/84 both negatively charged and positively charged domains of the protein were removed. Removal of the C-terminal negatively charged domain increased DNA binding activity of the protein, whereas removal of both domains decreased DNA binding activity of the protein (15). Table I shows that for both mutant proteins the end-to-end distance of DNA in protein-DNA complex was the same as for the wild type protein, suggesting that the conformation of DNA in the complex with the wild type protein and with the deletion mutants was the same.

The Effect of cHMGI Binding on DNA Conformation—FRET experiments identical to those described above for cHMG1a were performed with cHMGI. In contrast to cHMG1a, luminescence decays with and without the cHMGI protein were essentially single-exponential and the lifetimes were not significantly changed in the presence of the protein. Removal of the C-terminal negatively charged domain increased DNA binding activity of the protein, whereas removal of both domains decreased DNA binding activity of the protein (15). Table I shows that for both mutant proteins the end-to-end distance of DNA in protein-DNA complex was the same as for the wild type protein, suggesting that the conformation of DNA in the complex with the wild type protein and with the deletion mutants was the same.

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\text{FIG. 4. Decays of sensitized acceptor fluorescence in donor-acceptor 30-bp DNA in the absence of cHMG1a (A), with 250 nm cHMG1a (B), and with 1100 nm cHMG1a (C). The DNA concentration was 120 nm. The solid lines in panels B and C correspond to nonlinear regression fits of the decay data to a double-exponential function (a third small amplitude (about 2% of the total amplitude) component with a lifetime fixed at the value measured by a donor decay in the absence of the protein (Fig. 3A) was also included in these fits). The double-exponential fitting revealed two components: a short-lived component (\( r = 20-40 \mu s \)), which was not analyzed further (see "Results") and a longer-lived component (\( r = 209 \mu s \) for the data in panel B, and \( r = 201 \mu s \) for the data in panel C), for which lifetime was essentially identical to an additional decay component appearing in donor decays in the presence of cHMG1a (Fig. 3, B and C).}
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\text{FIG. 5. Decay of donor luminescence in an equimolar mixture of 30-bp DNA duplexes labeled with donor-only and acceptor-only, respectively. A, no protein; B, 250 nm cHMG1a; C, 1100 nm cHMG1a. The DNA concentration was 240 nm (total concentration). Solid lines represent the best fit of the decay data to a single-exponential function. The fitted lifetimes in the absence and in the presence of the protein were identical within the experimental error.}
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ter suited for measuring longer distances. The $R_e$ value for donor-acceptor DNA in 65% D$_2$O was 61.5 Å (12% increase compared with water). Fig. 6 shows the decay curves for 30-bp donor-acceptor DNA in the absence (Fig. 6A) and presence of 3.3 μM cHMGI (saturating concentration; see Fig. 1C). In both cases the decays could be fitted to a single-exponential function. The lifetime of the donor in donor-only 30-bp DNA was 1181 μs and was reduced to 1154 μs upon binding of cHMGI. The lifetime in donor-only 30-bp DNA was 1236 μs (data not shown). In accordance with the increased $R_e$, the energy transfer in free DNA was increased to 0.044 (corresponding to ~103 Å) compared with the measurement in 100% H$_2$O. Binding of cHMGI produced a small but measurable increase in energy transfer (to 0.065) and corresponding decrease in end-to-end distance (to ~96 Å). These data suggest that binding of cHMGI induces minor deformation of DNA.

**DISCUSSION**

**DNA Bending Angle Induced by cHMGIa**—FRET end-to-end distance measurements in a 30-bp DNA fragment revealed that binding of cHMGIa to DNA resulted in a major conformational change in DNA. Using the measured end-to-end distance in the cHMGIa-DNA complex (~50.5 Å), it is possible to estimate the degree of bending induced by cHMGIa (Fig. 7).

When calculating the bend angle from FRET measurements, it is important to consider the helical nature of double-stranded DNA, which determines the relative position of fluorochromes along the double helix. Clegg et al. (32) presented experimental data illustrating the influence of helical geometry of DNA on FRET distance measurements and presented a simple model permitting interpretation of FRET measurements in double-stranded DNA. In this model, the relative positions of fluorochrome probes attached covalently to DNA molecule is described by a set of three parameters: $L$, representing the distance along the helix axis between donor and acceptor if they would be attached to the same base pair, $d$ and $a$, representing a distance by which donor (or acceptor) extends perpendicularly to the axis of the helix, and $\phi$, representing the angle between donor and acceptor when they are separated by 1 base pair. Since the chemistry and the linkers used to attach fluorochromes in our study were similar to those used by Clegg et al. (32), we expected that similar values of the above parameters should be applicable in both cases. Analysis of FRET data for europium chelate-CY5 donor-acceptor pair using a series of DNA fragments differing in length (data not shown) demonstrated that indeed it was the case. The parameters that best described our data were: $L = 9$ Å, $d = a = 12$ Å (in calculating $L$, $d$, and $a$, we used $\phi = 220^\circ$, a value determined by Clegg et al. (32), since in both studies the probes were attached to 5' phosphate of the opposite strands and thus this angle should be the same in both cases). For comparison, these parameters in the above study were: $L = 13$ Å, $d = 19$ Å, and $a = 13$ Å (32). These parameters allow us to predict that in the 30-bp DNA used in this study, donor and acceptor would be located on opposite sides of the helix, extending approximately 2 Å from the surface of the helix (Fig. 7).

Using the above model parameters, it is possible to estimate the degree of DNA bending consistent with the end-to-end distance of 50.5 Å observed in cHMGI1a-DNA complex. Assuming that in the bent DNA the probes would remain on opposite sides of the helix, the calculated bending angle would be about 170° (Fig. 7). However, it is likely that the deformation of DNA by cHMGIa will include some untwisting of DNA in a region of a direct protein-DNA contact. Such untwisting is suggested by a structural analysis of other proteins, which share with cHMGIa properties of inducing a sharp bend in DNA and binding to the minor groove. TBP protein induces about 100° bend, binds to drastically deformed and widened minor groove, and causes the loss of ~1/3 of a turn of the helix (120° untwisting) (1). The DNA binding domain of SRY protein causes an 80° bend in DNA and about 100° untwisting (8). The DNA binding domain of LEF-1 transcription factor induces about 120° bend and significant untwisting (7). SRY and LEF-1 proteins are specific DNA-binding proteins containing HMG1-BD. It is thus likely that DNA bending induced by cHMGIa will be accompanied by DNA untwisting in the range of around 90°. Untwisting of DNA in cHMGIa-DNA complex will result in a change in a relative position of donor and acceptor molecules, as illustrated in Fig. 7. Untwisting by 90° would position the fluorochromes such that 50.5 Å end-to-end distance would correspond to a 150° bend (Fig. 7). Assuming untwisting by 180°, a 135° bend can be calculated (Fig. 7). By analogy to TBP, SRY, and LEF-1, we believe that the most probable bend would be the one obtained assuming about 90° untwisting, i.e. 150°. Thus, cHMGIa binding induces a rather remarkable DNA bending. Although DNA bending by nonspecific HMG1 protein has not been measured previously, bovine HMG1 produces DNA circles that could be formed in ligase-mediated circularization assay with DNA as short as 59 bp (10). This observation is consistent with a sharp DNA bending observed by us using the FRET method.

The assumption that the bending induced by cHMGIa involves about 90° of DNA untwisting is further supported by the
The HMG1/2 proteins belong to the most abundant chromosomal non-histone proteins. In rat tissues the concentrations of HMG1/2 proteins was estimated to be 1 molecule/2–3 nucleosomes (33), while in insect cells of Chironomus 1 cHMGIa molecule/5–6 nucleosomes occurs (34). Since indirect immunofluorescence staining of polytene chromosomes of Chironomus revealed that the patterns of the cHMGIa staining apparently follow the DNA staining pattern (34), it is likely that in interphase nucleus the majority of cHMGIa molecules are bound to DNA. This observation, together with the strong DNA bending activity of cHMGIa and its relative abundance, suggests that this protein plays a very important role in modulation of chromatin structure. It seems that in comparison to cHMGIa protein, only the nucleosome octamers have a greater cumulative DNA bending potential. Thus, cHMGIa, along with other members of a class of highly abundant HMG1/2, could be a pivotal organizer of the DNA-packaging in the cell.

The regions close to the C terminus of the HMG1-BD strongly influence the DNA-binding affinity of HMG1/2 proteins (11, 15, 35, 36). Alterations of the binding affinity by 2 orders of magnitude were observed in studies employing protein mutants (15) or C-terminally phosphorylated (37) cHMGIa. These dramatic changes in the binding affinity apparently did not influence the extent of the induced DNA bend. In contrast, the positively charged C-terminal extension of the HMG1-BD of hLEF-1 increased the angle of DNA bend (6). These differences are probably due to the structural diversity in the protein-DNA complexes of cHMGIa and hLEF-1. In LEF-1 DNA complexes, the C-terminal region of HMG1-BD binds across the narrowed major groove of the DNA, making extensive contacts with the sugar-phosphate backbone (7), whereas in cHMGIa (38) and A-Domain in HMG1 (39), this region of HMG1-BD (Helix III) is not involved or only marginally involved in the protein-DNA interaction. The spatial arrangement of the C-terminal regions in the HMG1 and cHMGIa proteins and in their complexes with DNA remains still unknown. Therefore, further studies in which intramolecular distances within these proteins and the protein-DNA complex would be determined are necessary to understand the mechanism of bend induction by proteins containing HMG1-BD.

**DNA Bending Angle Induced by cHMGI**—The changes in end-to-end distance induced by cHMGI binding are very small, and since the measured distances are long, the precision of these measurements is rather low. It is therefore impossible to analyze the effects of cHMGI binding on DNA conformation in detail comparable with that possible in the case of cHMGIa. The measured decrease of end-to-end distance from ~103 Å to about ~96 Å suggests only that the bending induced by cHMGI most likely is less than 40°. This rather moderate effect of cHMGI binding on the DNA conformation was also supported by the ligase-mediated circularization assay in which cHMGI protein was found to be unable to facilitate formation of DNA circles. As the mammalian HMGI protein has been also shown to induce only subtle bends in the DNA (23), our results may indicate that both mammalian and insect HMGI proteins have similar cellular function(s).

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