Basic and Acidic Regions Flanking the HMG Domain of Maize HMGa Modulate the Interactions with DNA and the Self-Association of the Protein†

Christoph Ritt,‡ Rudi Grimm,§ Silvia Fernández,|| Juan C. Alonso,|| and Klaus D. Grasser*,‡

Institut für Biologie III, Albert-Ludwigs-Universität Freiburg, Schänzlestrasse 1, D-79104 Freiburg, FRG, Hewlett-Packard GmbH, Hewlett-Packardstrasse 8, D-76337 Waldbronn, FRG, and Centro Nacional de Biotechnología, CSIC, Campus de la Universidad Autónoma de Madrid, Cantoblanco, 28049 Madrid, Spain

Received October 22, 1997; Revised Manuscript Received December 8, 1997

ABSTRACT: The maize HMGa protein is a typical member of the family of plant chromosomal HMG1-like proteins. The HMG domain of HMGa is flanked by a basic N-terminal domain characteristic for plant HMG1-like proteins, and is linked to the acidic C-terminal domain by a short basic region. Various derivatives of the HMGa protein were expressed in *Escherichia coli* and purified. The individual HMG domain can functionally complement the defect of the HU-like chromatin-associated Hbsu protein in *Bacillus subtilis*. The basic N-terminal domain which contacts DNA enhances the affinity of the protein for linear DNA, whereas it has little effect on the structure-specific binding to DNA minicircles. The acidic C-terminal domain reduces the affinity of HMGa for linear DNA, but does not affect to the same extent the recognition of DNA structure which is an intrinsic property of the HMG domain. The efficiency of the HMGa constructs to facilitate circularization of short DNA fragments in the presence of DNA ligase is like the binding to linear DNA altered by the basic and acidic domains flanking the HMG domain, while the supercoiling activity of HMGa is only slightly influenced by the same regions. Both the basic N-terminal and the acidic C-terminal domains contribute directly to the self-association of HMGa in the presence of DNA. Collectively, these findings suggest that the intrinsic properties of the HMG domain can be modulated within the HMGa protein by the basic and acidic domains.

HMG1-like[1] proteins are a family of abundant chromosomal nonhistone proteins which bind DNA nonsequence-specifically. Proteins belonging to this family have been identified in a wide variety of eukaryotic organisms (*1*). HMG1-like proteins contain either 1 (plants, insects, and yeast) or 2 (vertebrates) copies of a common DNA-binding motif of approximately 75 amino acid residues termed the HMG domain (*2, 3*). This domain has a characteristic L-shaped fold formed by three α-helixes with an angle of ∼80° between the two arms (*4*−*7*). DNA binding occurs primarily by contacts between the minor groove of DNA (*8, 9*) and the concave surface of the HMG domain (*10*−*15*).

Outside the relatively conserved HMG domain, HMG1-like proteins of different organisms display a considerable structural variability (*16*). Thus, there is no acidic C-terminal domain in the yeast NHP6A/B proteins (*17*), a short acidic tail in the corresponding insect proteins (*18, 19*), and significantly longer acidic tails in vertebrate HMG1/2 (*20*) and some plant HMG1-like proteins (*16*). Similarly, the length of the basic region linking the acidic C-terminal domain to the HMG domain is relatively variable. In some insect and vertebrate HMG1-like proteins, the regions flanking the HMG domain have been shown to modulate the general affinity of the protein for DNA (*21*−*24*).

Despite the differences in primary structure, the HMG1-like proteins of various species share several features in their DNA-binding properties. They bind selectively to distorted DNA structures such as four-way junction DNA (*25, 26*), cisplatin-modified DNA (*27*−*29*), and DNA-minicircles (*24, 30, 31*). In addition, the recognition of deformable DNA sequences might contribute to the target site selection of these proteins (*32*−*34*). HMG1-like proteins can bend linear duplex DNA (*24, 30, 31, 35, 36*) and constrain negative supercoils in plasmid DNA (*22, 37*−*39*). They can stimulate the binding of certain transcription factors to their specific target sites (*40*−*42*), and can enhance transcriptional activation (*43*−*46*). Furthermore, HMG1-like proteins facilitate the formation of complex nucleoprotein structures in certain recombination reactions (*35, 47*−*50*).

The HMGa protein (*51*) is the most abundant of the four maize HMG1-like proteins (*39, 52*) and has a distinctive overall structure typical for the plant proteins belonging to the HMG1 family (*16*). Accordingly, the HMG domain of HMGa is flanked by an N-terminal basic region, and the

† Supported by Grants PB 96-0817 from DGCICYT and 06G/004/96 from the Consejería de Educación y Cultura de la Comunidad de Madrid to J.C.A., and by Grant Gr 1159/4-4 from the Deutsche Forschungsgemeinschaft to K.D.G. C.R. is supported by a Graduierten Kolleg fellowship and K.D.G. by a Habilitations fellowship from the Deutsche Forschungsgemeinschaft.

* Corresponding author. Fax: 49-761-203 2745. E-mail: grasser@biologie.uni-freiburg.de.

‡ Albert-Ludwigs-Universität Freiburg.

§ Hewlett-Packard GmbH.

|| Campus de la Universidad Autónoma de Madrid.

[1] Abbreviations: HMG protein, high mobility group protein; DSS, disuccinimidyl suberate; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide; MMS, methyl methanesulfonate; 4NQO, 4-nitroquinoline-1-oxide.

S0006-2960(97)02620-2 CCC: $15.00
Published on Web 02/06/1998

acidic C-terminal domain is linked by a short basic region to the HMG domain. We have examined here the contribution of the regions flanking the HMG domain of the maize HMGa protein on the in vitro interactions with DNA and the self-association of the protein. Furthermore, we have analyzed the ability of HMGa to complement the phenotype of a mutant *B. subtilis* strain impaired in the chromatin-associated HU-like Hbsu protein in vivo.

EXPERIMENTAL PROCEDURES

Expression, Purification, and Characterization of the Proteins. Distinct regions of maize HMGa cDNA (*51*) were amplified by PCR using the following primers: P1: 5′-CTCGGATCCCATATGAAGGGGGCCAAATCCAAGG and P2: 5′-AATTAAGCTTACTCGTCATCATCTTCATCCTCC for full-length HMGa(M1-E157); P1 and P3: 5′-TCTTCAAGCTTGGCAGGAGCC for HMGa(M1-K123); P1 and P4: 5′-GCTCTCGCCAAGCTTGTAGGC for HMGa-(M1-Y109); P5: 5′-GGGGGATCCGGCAAGGACC and P2 for HMGa(G35-E157); P5 and P3 for HMGa(G35-K123); P5 and P4 for HMGa(G35-Y109).

The obtained PCR fragments were digested with *Bam*HI and *Hin*dIII, and cloned into the expression vector pQE9cm (*39*). The plasmid pQE9cm-HMGa(M1-D134) was constructed by digesting the PCR fragment obtained with primers P6 (5′-GGGCGAGAGCACTGCAG) and P7 (5′-AATTAAGCTTAGTCAGACTCCTCTTCATCTTC) with *Pst*I and *Hin*dIII, and cloning it into pQE9cm-HMGa(M1-E157) digested with *Pst*I and *Hin*dIII. The resulting plasmids provide an N-terminal 6xHis-tag suitable for Ni-NTA−agarose affinity purification (Qiagen). The PCR fragment obtained with primers P1 and P2 (full-length HMGa) was also digested with *Nde*I and *Hin*dIII, and cloned into the expression vector pT7cm [created by replacing the ampicillin resistance of pT7-7 (*53*) by the chloramphenicol resistance of pBC-SK (Stratagene)] to express HMGa without 6xHis-tag. All plasmids were checked by DNA sequencing. Expression of the proteins, and the purification of 6xHis-tagged HMGa(M1-E157), HMGa(G35-E157), and HMGa-(M1-D134) by three-step column chromatography (Ni-NTA−agarose, S Sepharose Fast Flow, ResourceQ), was as described for maize HMGc (*39*). The final purification step of HMGa(M1-K123), HMGa(M1-Y109), HMGa(G35-K123), and HMGa(G35-Y109) was different. To the fractions eluted from the S Sepharose Fast Flow FPLC column containing the respective protein was added solid $(NH_4)_2SO_4$ to a final concentration of 1.8 M. The solution was centrifuged (15000*g*, 15 min) and the supernatant applied to a phenyl-Sepharose FPLC column (Pharmacia) equilibrated with buffer D (10 mM sodium phosphate pH 7.0, 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF) containing 1.8 M $(NH_4)_2SO_4$. After the column was washed with this buffer, proteins were eluted with a linear gradient of 1.8−0 M $(NH_4)_2SO_4$ in buffer D. The fractions containing pure HMGa constructs were identified by SDS−PAGE, and the $(NH_4)_2SO_4$ was removed using PD-10 columns (Pharmacia). For a comparison, we expressed full-length HMGa without a 6xHis-tag in *E. coli* using the plasmid pT7cm-HMGa. This protein was purified by S Sepharose Fast Flow and ResourceQ chromatographies as described above. In none of the assays used in this report did we observe differences between the 6xHis-tagged HMGa and the HMGa protein without 6xHis-tag. All the purified proteins were characterized by SDS−PAGE, N-terminal sequencing, and MALDI-TOF mass spectrometry as described in (*39*), and by CD spectrometry in 10 mM sodium phosphate, pH 7.0, 0.5 mM EDTA, and 0.5 mM DTT as described in (*14*).

PCR was used as described above to amplify the DNA sequences corresponding to full-length HMGa (M1-E157) and the truncated derivatives (M1-K123, G35-Y109, G35-E157), except that a primer with extra residues showing homology to the consensus ribosomal binding site for *B. subtilis* was used at the 5′-end. The amplified DNA segments were cloned into *Eco*RI−*Hin*dIII cleaved pHP13 (*54*) to render plasmids pCB205, pCB175, pCB174, and pCB204, respectively. The plasmid-borne *hbs* gene (pCB188) has been previously described (*55*). The derivatives of plasmid pHP13 have ∼5 copies per cell (*54*; data not shown). Treatment of the *B. subtilis* strains BG397 (*hbs*47) and BG405 (*hbs*4755) with MMS (Eastman Kodak) or 4NQO (Sigma) was performed as described (*55*).

DNA−Cellulose Binding. HMGa constructs (10 μg) were bound to DNA−cellulose (Sigma, 3 mg of calf thymus dsDNA/g of cellulose, length on average 20 kbp) equilibrated with 10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 0.1 mM DTT, and 30 μg/mL BSA (*56*). DNA−cellulose was collected by centrifugation through disposable mini columns (Promega). The bound proteins were eluted with stepwise increasing NaCl concentrations in equilibration buffer, and precipitated with 25% (w/v) TCA. The precipitated proteins were collected by centrifugation (25000*g*, 20 min), and the pellets were washed twice with acetone, resuspended in SDS-loading buffer, and analyzed by SDS−PAGE. The proteins were quantified from the Coomassie-stained gels using the Digital Video System (Polaroid) and NIH Image software.

Tryptic Digest of HMGa and Analysis of the Peptides. Limited digests of HMGa (10 μg) were carried out in the absence or presence of supercoiled pBluescript (10 μg) with 0.2% (w/v) trypsin at 20 °C for 15 min in buffer D. The digestion was stopped by addition of 2% formic acid, and peptide mapping was performed on a 1 × 250 mm Vydac C-18 column using the HP 1100 HPLC system (Hewlett-Packard). Peptides were eluted using a water/acetonitrile gradient (1−45%, in 75 min at 40 °C, at a flow rate of 50 μL/min). One microliter aliquots of the peptide fractions were analyzed by MALDI/TOF mass spectrometry using dihydroxybenzoic acid as matrix on the HP G2025A MALDI/TOF system (Hewlett-Packard). The residual fractions were analyzed by peptide sequence analysis using the HP G1005A protein sequencing system (Hewlett-Packard).

Electrophoretic Mobility Shift Assay. DNA-binding assays with various amounts of the HMGa constructs and the linear and circularized 78-bp fragment were performed as described previously (*39*).

Circularization Assay. A 20 μL reaction mix containing 30 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 10 mM DTT, 0.5 mM ATP, the ^{32}P-end-labeled 116-bp DNA fragment [obtained by digesting pBluescript-SK with *Bss*HII/*Xba*I, and creating blunt ends using the Klenow fragment of DNA polymerase I], and various concentrations of HMGa constructs was incubated for 10 min, before 1.2 units of T4 DNA ligase (Fermentas) was added. The ligase reaction was terminated after 30 min at 15 °C by shifting the temperature to 70 °C for 15 min. Some samples were incubated with

60 units of exonuclease III (Fermentas) at 37 °C for 30 min, and then extracted with phenol/chloroform. The deproteinized DNA was analyzed by electrophoresis in 5% polyacrylamide gels in 0.5 × TBE, and the dried gels were autoradiographed.

Supercoiling Assay. Supercoiling assays with relaxed, closed circular pBluescript in the presence of vaccina virus topoisomerase I (provided by Dr. K. Schnetz) were performed as described previously (*39*).

Protein/Protein Cross-Linking. For lysine-specific cross-linking, the HMGa constructs were treated with 0.25 mM of the homobifunctional reagent suberic acid bis(*N*-hydroxysuccinimide ester) (=disuccinimidyl suberate, DSS) (Sigma) from a freshly prepared stock of 25 mM in DMSO (*56*). Cross-linking was performed at 21 °C in the absence or presence of supercoiled pBluescript in 50 mM Hepes/NaOH pH 8.0, 0.1 mM EDTA, 10 mM $MgCl_2$, 0.5 mM DTT. Reactions were stopped by precipitation of the proteins with 25% (w/v) TCA as described above. The proteins were analyzed by SDS−PAGE and Coomassie staining.

For cross-linking of carboxyl with amino groups, the HMGa constructs were treated with 5 mM 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) (Pierce) from a freshly prepared stock of 100 mM in H_2O. Cross-linking was performed at 21 °C in the absence or presence of supercoiled pBluescript in 10 mM sodium phosphate, pH 7.0, 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF. Reactions were stopped and the proteins analyzed as described above.

RESULTS AND DISCUSSION

Production and Characterization of Recombinant HMGa Constructs and Their Affinity for DNA−Cellulose. To examine the role of the basic and acidic regions flanking the HMG domain of HMGa in their interactions with DNA and in the self-association of the protein, different HMGa constructs were expressed in *E. coli* and purified (Figure 1A): the full-length protein HMGa(M1-E157); HMGa with a truncated acidic tail, HMGa(M1-D134); HMGa lacking the acidic tail, HMGa(M1-K123); HMGa lacking the acidic tail and the basic linker region, HMGa(M1-Y109); HMGa lacking the basic N-terminal domain, HMGa(G35-E157); HMGa lacking the basic N-terminal domain and the acidic tail, HMGa(G35-K123); and the individual HMG domain, HMGa(G35-Y109). The purified recombinant proteins were characterized by SDS−PAGE (Figure 1B), MALDI/TOF mass spectrometry, and N-terminal sequencing (data not shown).

The relative affinities of the HMGa constructs for long double-stranded calf thymus DNA immobilized on cellulose were measured. The proteins were bound to the DNA−cellulose in the absence of NaCl, eluted with stepwise increasing NaCl concentrations, analyzed by SDS−PAGE, and quantified (Figure 2A). Full-length HMGa (M1-E157) and the HMG domain (G35-Y109) display an almost identical affinity for the DNA−cellulose. The absence of the acidic C-terminal domain (M1-K123) significantly enhances the affinity of the protein, whereas the absence of the basic N-terminal domain (G35-E157) reduces the affinity to a comparable extent, suggesting that the positive effect of the basic N-terminal domain and the negative effect of the acidic C-terminal domain are balanced in HMGa. Basic and acidic regions flanking the HMG domain in vertebrate

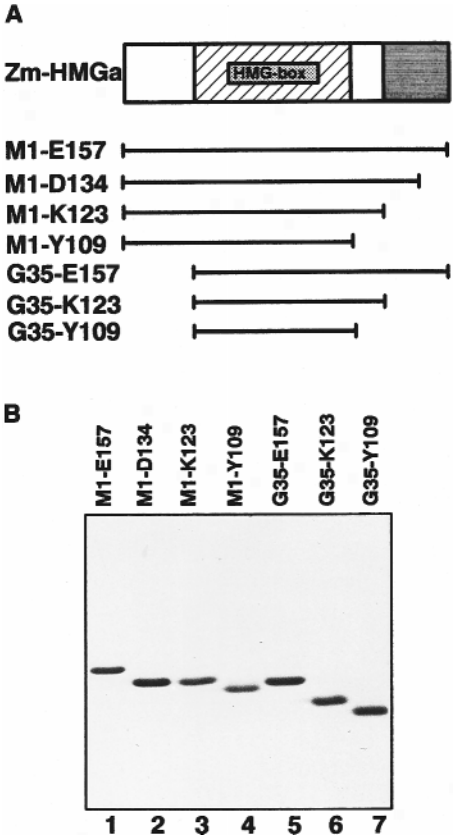

FIGURE 1: Schematic representation of the recombinant constructs of the maize HMGa protein and SDS−PAGE analysis of the corresponding proteins. (A) Schematic representation of the domain structure of the HMGa protein from maize (*Zea mays*). The HMG domain is indicated by a hatched box, the acidic C-terminal domain by a shaded box, and the basic N-terminal domain and the basic linker region by open boxes. The protein constructs of HMGa that were used in this study are shown below, and the relevant amino acid positions are indicated. (B) SDS−PAGE of the recombinant HMGa constructs that were expressed as 6xHis-tagged proteins in *E. coli* and purified.

and insect HMG1-like proteins that differ from the corresponding regions of plant HMG1-like proteins have been reported to modulate the general affinity for DNA positively or negatively, respectively (*22*−*24*, *56*). In view of the severe loss of α-helicity in the central HMG domain (B domain) of HMG1, when the N-terminal HMG domain (A domain) is absent (*37*, *57*, *58*), we compared the far-UV CD spectra of full-length HMGa and the construct lacking the basic N-terminal domain (Figure 2B). The similar negative peak at 222 nm in the CD spectra indicates no significant loss of helical structure in (G35-E157) when compared with the full-length protein (M1-E157), suggesting that the relatively low affinity of (G35-E157) for the DNA−cellulose might not be caused by structural distortion of the protein. The negative effect of the acidic tail on DNA−cellulose binding appears to be a nonspecific electrostatic effect, since examination of chimeric HMGa proteins containing (instead of the HMGa acidic tail) the acidic tail of rat HMG1 (*59*) or *Chironomus* cHMG1a (*19*) that have a higher or lower negative charge, respectively, reveals that the reduction of the affinity for DNA−cellulose does not require a specific amino acid sequence, and that it is correlated to the negative charge of the acidic tail (data not shown). Accordingly, the negative effect of the acidic tail is less prominent with the construct (M1-D134) containing a truncated acidic tail, when

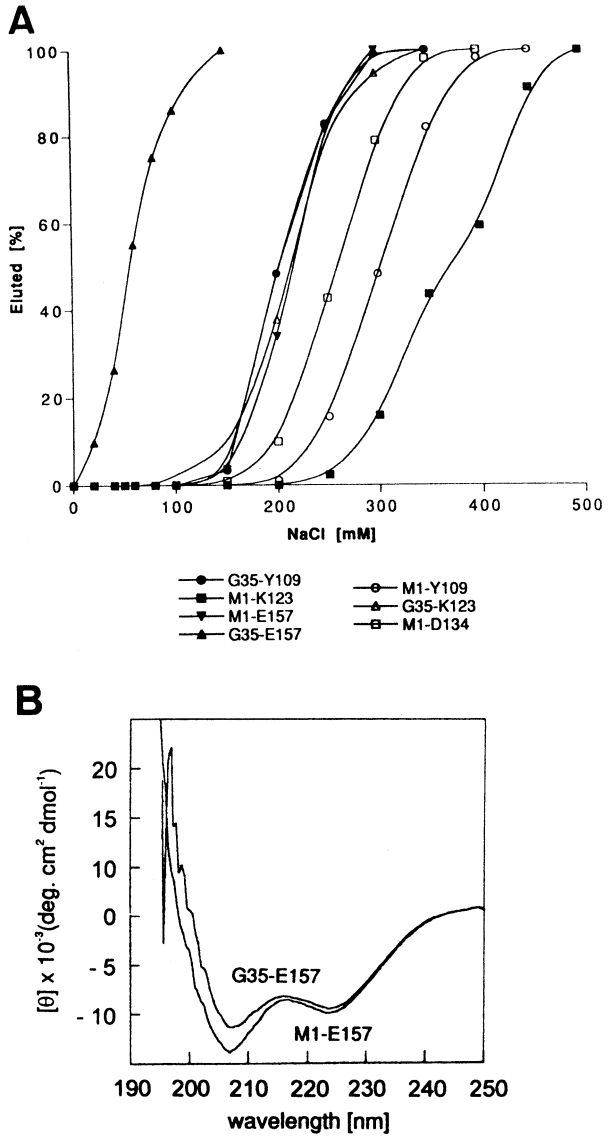

FIGURE 2: (A) The HMGa constructs interact with different affinities with DNA−cellulose. The different HMGa constructs (the respective symbols are indicated) were bound to DNA−cellulose and eluted with stepwise increasing NaCl concentrations. The eluted proteins were analyzed by SDS−PAGE and quantified. Each curve represents the average of at least four different experiments. (B) Far-UV CD spectra of HMGa(M1-E157) and HMGa(G35-E157). The spectra were recorded at ~20 °C with measurements every 0.5 nm.

compared with the full-length protein (M1-E157) (Figure 2A). Unlike the vertebrate and insect proteins (*23, 24, 56*), the comparatively less basic region of HMGa (N110-K123; net charge +3) linking the HMG domain to the acidic tail has only little effect on the affinity for DNA−cellulose [cf. (G35-Y109) and (G35-K123)]. In the following experiments of this report, we will focus mainly on the four constructs: full-length HMGa (M1-E157), the HMG domain (G35-Y109), the construct lacking the acidic C-terminal domain (M1-K123), and the construct lacking the basic N-terminal domain (G35-E157), since these proteins gave the most significant results (see below).

Involvement of HMGa Protein in Recombination-Dependent DNA Repair. The loss of the chromatin-associated HU protein in *E. coli* is not lethal, but a large percentage of cells in a growing population lose their chromosome. Previously, it had been shown that the expression of the yeast HMG domain proteins, the nuclear NHP6A, or the mitochondrial HM, in mutant *E. coli* cells lacking the structurally unrelated HU protein, partially restored the normal morphological appearance of these cells, specifically in nucleoid condensation and segregation (*60, 61*). Both yeast HMG1-like proteins lack an acidic C-terminal domain. The *B. subtilis hbs* gene encodes an essential chromatin-associated HU-like protein termed Hbsu. A leaky mutation (*hbs*4755) in the Hbsu gene product (F47W, R55A) renders cells very sensitive to the lethal effect of methyl methanesulfonate (MMS) or 4-nitroquinoline-1-oxide (4NQO), when compared to the control (*hbs*47) strain (F47W) (*55*).

To examine whether HMGa is involved in recombination-dependent DNA repair, we expressed full-length HMGa (M1-E157), the HMG domain (G35-Y109), the construct lacking the basic N-terminal (G35-E157), and the construct lacking the acidic C-terminal (M1-K123) domain in the *B. subtilis hbs47* (BG397) and *hbs4755* (BG405) strains. The involvement of the HMGa protein and its derivatives in recombination-dependent DNA repair was analyzed by exposing the cells to the lethal effect of 10 mM MMS or 100 mM 4NQO. The *B. subtilis hbs*47 strain bearing plasmid or plasmid-free was as sensitive to 10 mM MMS as was the *hbs*$^+$ strain (data not shown), reinforcing the 'wild-type' characteristics of the Hbsu F47W derivative (*55*). The increased sensitivity of the double mutant strain *hbs*4755 to the lethal effect of both MMS (Figure 3) and 4NQO (data not shown) is not affected by the presence of the control plasmid vector (data not shown). The 10 mM MMS dosage required to kill 90% of BG405 cells (DL_{10}) is about 15 min when compared to *hbs*47 or wild-type *hbs*$^+$ cells (> 150 min) (Figure 3A). The presence of plasmid-borne Hbsu or of the HMG domain (G35-Y109) in the BG405 background (*hbs*4755) results in the recovery of the MMS^s phenotype (DL_{10} > 150 min) (see Figure 3B). The presence of the HMGa constructs (M1-E157), (M1-K123), and (G35-E157) only partially complements the defect of *hbs*4755 cells (DL_{10} 58, 50, and 67 min, respectively) when compared to plasmid-free cells (DL_{10} 15 min). After 45 min of exposure of the BG405 (*hbs*4755) strain to 10 mM MMS, the viability drops about 30−35-fold. The viability of the BG405 strain drops only about 1.6−2-fold when Hbsu or the HMG domain are present, and about 5−6-fold when the HMGa constructs (M1-E157), (M1-K123), or (G35-E157) are present in the BG405 background (see Figure 3B). Similar results were observed when the cells were exposed to the lethal effect of 4NQO (data not shown). From these results, we can also infer that the truncations of the HMGa constructs do not lead to any gross distortion of the protein structure, when compared to the full-length HMGa protein.

The chromatin-associated HU, Hbsu, and HMG1-like proteins bind with high affinity to distorted double-stranded DNA structures, or to single-stranded DNA containing breaks or gaps (*28, 55, 62*−*64*). These distorted DNA structures are thought to appear during DNA recombination and/or DNA repair processes and are the target of DNA repair proteins. In bacteria, chromatin-associated proteins (e.g., *E. coli* HU or *B. subtilis* Hbsu) can facilitate DNA repair that involves the removal of DNA damages (*55, 64, 65*). In mammalian cells, however, HMG1 or a derivative without the acidic C-terminal domain could either shield the adducts

A

	DL_{10} incubation time [min]
BG397	>150
BG405	15
BG405 [Hbsu]	>150
BG405 [HMGa(M1-E157)]	58
BG405 [HMGa(G35-E157)]	67
BG405 [HMGa(M1-K123)]	50
BG405 [HMGa(G35-Y109)]	>150

B

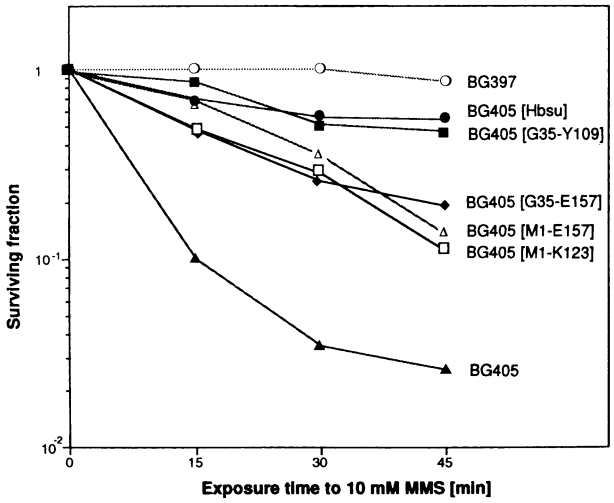

FIGURE 3: Functional complementation of the HMGa constructs in vivo. (A) DL_{10}, defined as the incubation time (in minutes) required to kill 90% of *B. subtilis* cells (BG397, *hbs*47; BG405, *hbs*4755) upon exposure to 10 mM MMS. (B) Survival of *B. subtilis* strains *hbs*47; *hbs*4755; *hbs*4755[pCB188-borne *hbs*]; *hbs*4755-[pCB174-borne (G35-Y109)]; *hbs*4755[pCB205-borne (M1-E157)]; *hbs*4755[pCB204-borne (G35-E157)]; and *hbs*4755[pCB175-borne (M1-K123)] following exposure to 10 mM MMS for the indicated time.

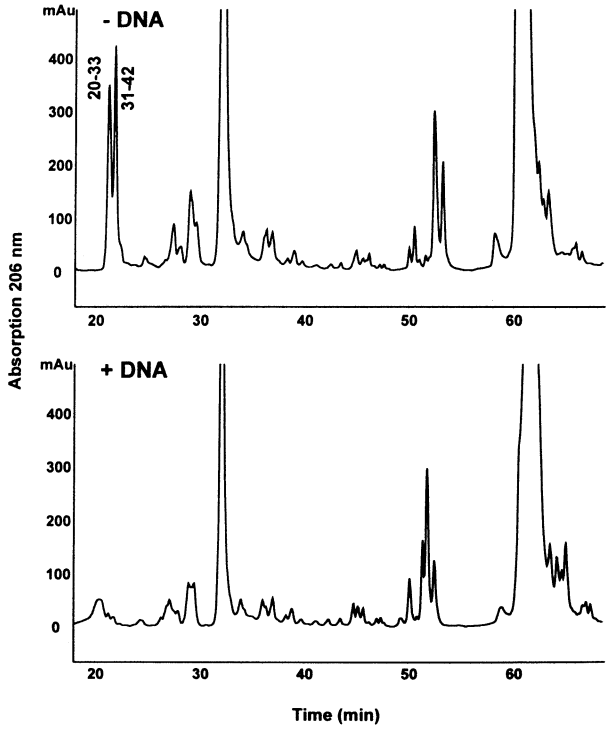

FIGURE 4: The basic N-terminal domain of HMGa is protected from tryptic digest when bound to DNA. Limited trypsin digests of HMGa (without the N-terminal 6xHis-tag) were performed either in the absence of DNA (top panel) or bound to supercoiled pBluescript (bottom panel), and the resulting peptides were separated by reversed-phase HPLC. The peptides were identified by MALDI/TOF mass spectrometry and peptide sequencing. The two peptides (S20-K33 and K31-K42) that are protected from tryptic digest in the presence of DNA are indicated.

from nucleotide excision repair (*66*−*68*) or inhibit the translesion synthesis of DNA cisplatin adducts (*69*). Our results show that the full-length HMGa protein (M1-E157) and derivatives without the basic N-terminal (G35-E157) or acidic C-terminal (M1-K123) domains partially complement, while the HMG domain (G35-Y109) fully complements the defect of *hbs4755* cells in the removal of alkyl groups or purine adducts generated by MMS and 4NQO, respectively. It is likely that the DNA-binding and -bending HMG domain of HMGa is (like Hbsu) involved in different DNA transactions in *B. subtilis*, including DNA repair (this work), transcriptional regulation (unpublished results), and DNA replication.

The Basic N-Terminal Domain of HMGa Is in Contact with DNA. In the previous section, we have shown that the HMG domain of HMGa is sufficient to complement the defect of a Hbsu mutant. However, the significant positive effect of the basic N-terminal region of HMGa on the affinity for DNA−cellulose (Figure 2A) suggested that this protein domain might be directly involved in DNA binding. Therefore, we performed limited trypsin digests of bona fide HMGa (without the 6xHis-tag) either free in solution or bound to supercoiled plasmid DNA, and analyzed the resulting peptides by reversed-phase HPLC (Figure 4). The peptides were identified by N-terminal sequencing and MALDI/TOF mass spectrometry. The most prominent difference between the two digests is the appearance of the two peptides S20-K33 and K31-K42, when HMGa was digested in the absence of DNA, indicating that in the presence of DNA this region of the protein is protected from protease cleavage by close contact with the DNA. The region S20-K33 is part of the basic N-terminal domain, whereas the region K31-K42 is part of the basic N-terminal domain and of the N-terminal extended strand of the HMG domain. The extended strand of various HMG domains has been shown previously to be critically involved in DNA binding (*11*, *13*−*15*, *36*). The basic N-terminal domain which is a distinctive feature of plant HMG1-like proteins (*16*) comprises residues M1-G35 in maize HMGa (net charge +10). This region of HMGa could interact with the sugar−phosphate backbone of DNA, thereby greatly extending the binding surface of the protein and enhancing the affinity for linear DNA, in a way similar to that suggested for basic regions situated C-terminally of HMG domains in other proteins (*13*, *23*, *24*, *56*, *70*, *71*).

Structure-Specific DNA Binding of the HMGa Constructs. The structure-specific DNA recognition of HMGa constructs was analyzed by comparing their binding to a circularized 78-bp DNA fragment relative to the corresponding linear 78-bp fragment of identical sequence. Increasing concentrations of the proteins were incubated with a mixture of the two ^{32}P-labeled DNA probes, and the formation of protein/DNA complexes was analyzed by electrophoretic mobility shift assays. Full-length HMGa (M1-E157), the HMG domain (G35-Y109), and the construct lacking the acidic tail (M1-K123) bind with a strong preference for the DNA

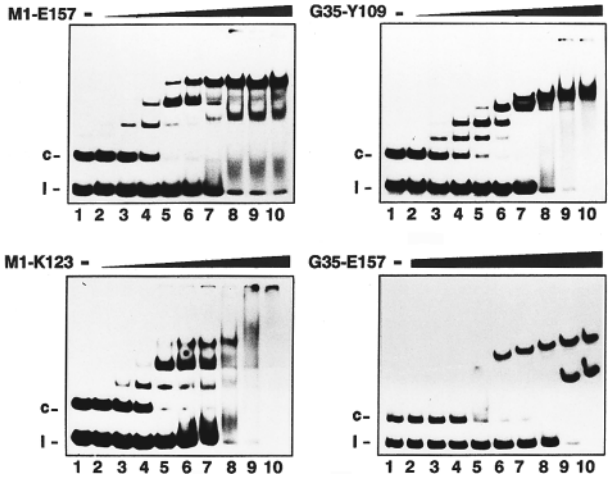

FIGURE 5: HMGa constructs bind with strong preference to a DNA minicircle relative to linear DNA. The indicated HMGa constructs were incubated at increasing concentrations (1 nM, lanes 2; 5 nM, lanes 3; 10 nM, lanes 4; 50 nM, lanes 5; 100 nM, lanes 6; 250 nM, lanes 7; 500 nM, lanes 8; 1 μM, lanes 9; 2 μM, lanes 10, for M1-E157, G35-Y109, and M1-K123); and (10 nM, lane 2; 50 nM, lane 3; 100 nM, lane 4; 250 nM, lane 5; 500 nM, lane 6; 1 μM, lane 7; 2 μM, lane 8; 5 μM, lane 9; 10 μM, lane 10, for G35-E157) with a ^{32}P-labeled 78-bp fragment and the ^{32}P-labeled 78-bp minicircle of identical sequence. The samples were analyzed by polyacrylamide gel electrophoresis and autoradiography. Migration positions of the linear and circularized 78-bp fragments in the absence of protein (lanes 1) are indicated by l and c, respectively.

minicircle in this assay, as they form 3−4 distinct complexes with the minicircle at 50−100-fold lower protein concentrations in comparison to the linear 78-bp fragment, demonstrating that the proteins interact structure-specifically with DNA (Figure 5). Interestingly, the absence of the acidic C-terminal domain does not enhance the affinity for the DNA minicircle as observed for DNA−cellulose (Figure 2A), indicating that the recognition of this DNA structure is an intrinsic property of the HMG domain. A very different behavior is observed for HMGa lacking the basic N-terminal domain (G35-E157) (Figure 5), since this construct has a ~50-fold reduced affinity for the DNA minicircle when compared with the other constructs. In addition, (G35-E157) forms only one complex with the minicircle which migrates increasingly slowly at increasing protein input, whereas the other constructs form several specific complexes. The abnormal migration of the complexes that (G35-E157) forms with the 78-bp minicircle might possibly be due to a different conformation of the DNA upon protein binding when compared with the other HMGa constructs.

The HMGa Constructs Differ in Their Ability To Modulate DNA Structure. We have used a ligase-mediated circularization assay with a short DNA fragment to analyze the ability of the HMGa constructs to promote DNA bending. Using a 116-bp DNA fragment, we analyzed the efficiency of various concentrations of the HMGa constructs to facilitate circularization of the fragment in the presence of T4 DNA ligase (Figure 6A). The identity of the circularized 116-bp fragment was evident from its resistance to exonuclease III treatment digesting linear products (cf. lanes 2 and 3), and from restriction enzyme digestion (data not shown). Whereas no circular products could be detected in the absence of the HMGa constructs, full-length HMGa, the HMG domain, and HMGa lacking the acidic tail facilitated the formation of 116-

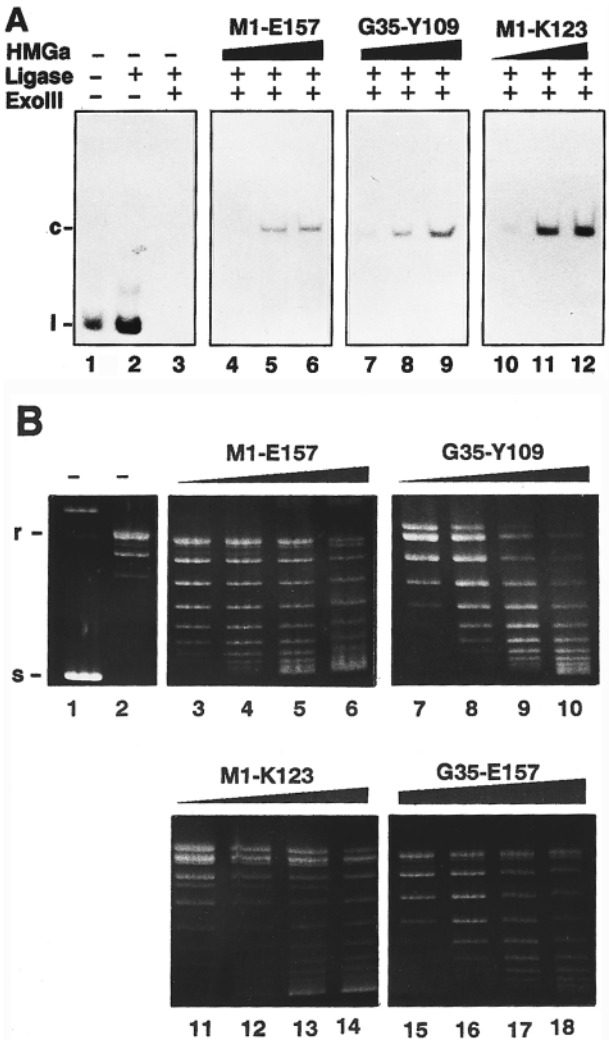

FIGURE 6: HMGa constructs differ in their ability to modulate DNA structure by bending and supercoiling. (A) Circularization assay with the HMGa constructs and a 116-bp DNA fragment. The ^{32}P-labeled 116-bp fragment was incubated with increasing concentrations of the indicated HMGa construct at protein concentrations of 100 nM (lanes 4, 7), 200 nM (lanes 5, 8), 400 nM (lanes 6, 9), and 10 nM (lane 10), 50 nM (lane 11), and 100 nM (lane 12). The addition of T4 DNA ligase and exonuclease III is indicated. Deproteinized reaction products were analyzed by polyacrylamide gel electrophoresis and autoradiography. The migration positions of the linear and circularized 116-bp fragment are indicated by l and c, respectively. (B) Supercoiling assay with plasmid DNA and the HMGa constructs in the presence of topoisomerase I. Relaxed, closed circular pBluescript was incubated in the presence of vaccina virus topoisomerase I with increasing amounts (protein:DNA molar input ratios: 10, 20, 40, 60 in lanes 3−6, 7−10, and 11−14; 50, 100, 200, 300 in lanes 15−18) of the indicated HMG construct. The deproteinized DNA was analyzed by agarose gel electrophoresis and ethidium bromide staining. The migration positions of relaxed (r) and supercoiled (s) plasmid DNA are indicated.

bp circles, albeit with different efficiencies: (M1-K123) > (G35-Y109) ≈ (M1-E157). In addition, (M1-K123) started to promote circularization of the DNA fragment at ~10-fold lower protein concentrations than (M1-E157) and (G35-Y109). Hence, the basic N-terminal domain enhances the DNA-bending activity which is an intrinsic property of the HMG domain, while the acidic tail has the opposite effect, as observed for the binding to linear DNA. The basic residues of the N-terminal domain could be envisaged as interacting with the inside of the bend, thereby neutralizing

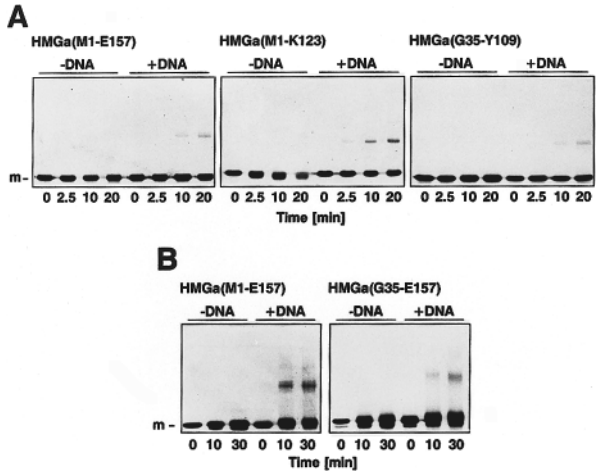

FIGURE 7: The basic N-terminal and the acidic C-terminal domain are involved in the self-association of HMGa. (A) The HMGa constructs (as indicated) either free in solution or bound to supercoiled pBluescript (100:1 protein:DNA molar input ratio) were treated with DSS for the times indicated, and analyzed by SDS−PAGE. (B) The HMGa constructs (as indicated) either free in solution or bound to supercoiled pBluescript (100:1 protein:DNA molar input ratio) were treated with EDC for the times indicated, and analyzed by SDS−PAGE. The migration positions of the monomeric proteins are indicated (m).

the charge of phosphate groups. Such an asymmetric phosphate neutralization could reduce repulsive interactions of neighboring phosphates, and enhance the HMG domain-induced bending (*72*, *73*). The DNA-bending activity of other HMG domain proteins is enhanced by basic regions situated C-terminally of the HMG domain (*56*, *71*). No circularization of the 116-bp fragment could be detected with (G35-E157), not even at 10-fold higher protein concentrations (up to 4 μM, data not shown), suggesting that the negative effect of the acidic C-terminal domain is even more prominent in the absence of the basic N-terminal domain. The negative effect of the acidic tail was also evident from circularization experiments with shorter DNA fragments, since full-length HMGa, for example, hardly mediated circularization of a 78-bp fragment, whereas HMGa lacking the acidic tail (and to a somewhat lower extent the individual HMG domain) efficiently caused circularization of the 78-bp fragment (data not shown).

The ability of the HMGa constructs to introduce supercoils into DNA was compared by incubating increasing concentrations of each protein with relaxed, closed circular plasmid DNA in the presence of vaccina virus topoisomerase I. All tested HMGa constructs display supercoiling activity in this assay (Figure 6B), and the introduced supercoils proved to be negative as evident from electrophoresis of the DNA in the presence of chloroquine (data not shown). Full-length HMGa (M1-E157), the HMG domain (G35-Y109), and the construct lacking the acidic tail (M1-K123) differ only slightly in their efficiency to supercoil the plasmid DNA (lanes 3−14). In contrast to these constructs, the supercoiling activity of HMGa lacking the basic N-terminal domain (G35-E157) is significantly reduced and requires ~10-fold higher protein input compared to the other HMGa constructs to induce comparable supercoiling (lanes 15−18).

The Basic and Acidic Domains Flanking the HMG Domain Are Involved in the Self-Association of HMGa. Electron microscopy revealed that HMG1 can form oligomeric beads when bound to DNA (*22*), and chemical cross-linking confirmed the self-association in the presence of DNA (*56*). We used the two cross-linking reagents disuccinimidyl suberate (DSS) and 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide (EDC) to examine the self-association of the HMGa constructs. The proteins, either free in solution or bound to supercoiled plasmid DNA, were treated with the homobifunctional lysine-specific cross-linking reagent DSS for various times (Figure 7A). In the absence of DNA, there was no evidence for cross-linked products, indicating that the HMGa constructs were monomeric in solution. We selected conditions of incomplete cross-linking that enabled us to resolve the appearance of the first cross-linked (dimeric) complex (seen above the monomers) in the presence of DNA, whereas more extensive cross-linking led to a ladder of cross-linked products (data not shown) similarly as observed with the HMG domains of HMG1 (*56*). When bound to DNA, the HMGa constructs were cross-linked by DSS to different extents, revealing the relative cross-linking efficiencies: (M1-K123) > (M1-E157) ≈ (G35-Y109). Marginal cross-linking could be detected with (G35-E157), however, only when 6-fold more extensive cross-linking was performed and the protein:DNA ratio was doubled (data not shown). The positive effect of the N-terminal basic domain on the cross-linking by DSS could be due to the greater proportion of lysine residues; however, the more efficient acylation of residues by the cross-linking reagent in (M1-K123) compared to (G35-Y109) is only evident in the absence of DNA (seen as increased electrophoretic mobility of protein monomers) possibly because the lysine residues are largely protected from modification in the presence of DNA. Alternatively, the more efficient cross-linking could result from a larger binding site when the basic N-terminal region is present enhancing the probability of protein/protein contacts.

To analyze possible interactions between carboxyl groups and amino groups, we treated the HMGa constructs in the presence and absence of DNA with the cross-linking reagent EDC. In the absence of DNA, there were again no cross-linked products detectable (Figure 7B). In the presence of supercoiled plasmid DNA, cross-linked products were formed with (M1-E157) and (G35-E157). No cross-linking occurred with the HMGa constructs lacking the acidic tail, indicating that the carboxyl groups of the glutamate and aspartate

HMGa construct	DNA-cellulose binding	minicircle binding	circularisation	supercoiling	DSS crosslinking	EDC crosslinking
M1-E157	+++	+++	+	+++++	++	+++
M1-D134	++++	+++	++	+++++	n. d.	n. d.
M1-K123	+++++	+++	+++	+++++	+++	-
M1-Y109	++++	+++	+++	+++++	n. d.	n. d.
G35-E157	+	+	-	+	-	+ +
G35-K123	+++	+++	++	+++++	n. d.	n. d.
G35-Y109	+++	+++	+	++++	+ +	n. d.

FIGURE 8: Summary of the relative activities of the HMGa constructs in the various in vitro assays.

residues of the C-terminal domain are crucial for EDC cross-linking of HMGa. Therefore, interactions of the acidic tail with basic regions of HMGa (HMG domain, N-terminal domain) are involved in the oligomerization of the protein. The acidic tail apparently interacts with the HMG domain and the basic N-terminal domain of HMGa, since full-length HMGa and the construct lacking the basic N-terminal domain are cross-linked by EDC, albeit with slightly different efficiency and pattern of cross-linked products. The basic part of the HMGa molecule (e.g., the HMG domain) has per se the ability to oligomerize in the presence of DNA (Figure 7A), as reported for the HMG domains of HMG1 (*56*, *74*), but as shown here interactions of the acidic C-terminal domain can contribute to the self-association of HMGa.

Possible Role of the Basic N-Terminal and the Acidic C-Terminal Domains in Modulating DNA Interactions and Self-Association of HMGa. The prokaryotic HU-like and the eukaryotic HMG1-like proteins share several features in their DNA-binding properties (*1*, *2*). The HMG domain of the HMGa protein can functionally complement the post-replication recombinational repair phenotype of the *B. subtilis* chromatin-associated Hbsu protein. The regions flanking the HMG domain significantly modulate some of the properties of the HMGa protein that are dominated by the characteristics of the HMG domain (Figure 8). The recognition of DNA structure and the supercoiling activity are only slightly influenced by the N-terminal and C-terminal domains, whereas they have considerable effects on the affinity for linear DNA, the DNA-bending activity, and the self-association of HMGa. In HMGa, the opposite effects of these basic and acidic domains are balanced, resulting in largely similar activities of full-length HMGa and of the isolated HMG domain. Interestingly, in the four maize HMG1-like proteins (HMGa, HMGc1/2, HMGd), the length and positive net charge of the basic N-terminal domain are correlated to the length and negative net charge of the acidic C-terminal domain (*39*, *51*, *52*). The construct lacking the basic N-terminal domain (G35-E157) displays exceptional properties in that it either has a very low activity in the assays used in this report or is entirely inactive. In view of our CD measurements and the in vivo complementation results, it is unlikely that the activity of this protein is impaired because of a severe structural distortion. The deletion of the basic domain, however, could lead to an imbalance within the molecule compared with full-length HMGa. Thus, in HMGa the basic N-terminal domain and the acidic C-terminal domain could be in direct contact, 'neutralizing' each other, which is suggested from electrostatic and steric reasons, since both domains protrude from the same side of the HMG domain and are presumably located in proximity to each other. Intramolecular interactions of HMGa could be modulated in vivo by other chromatin components. We propose that in (G35-E157) the 'natural partner' for the intramolecular interaction with the acidic tail is missing, and that the acidic tail might fold back to contact the basic HMG domain. Such an interaction between the acidic C-terminal domain and the HMG domain might account for the abnormal properties observed for (G35-E157); however, verification of that requires a detailed structural analysis. A similar hypothesis was put forward for HMG1 lacking the A domain (*37*, *57*, *58*), or for *Drosophila* HMG-D (*24*).

ACKNOWLEDGMENT

We thank Elke Brutzer and Monika Messerschmid for excellent technical assistance; Stefan Böcker and André Zimmermann for help with computer graphics; Drs. Jean Thomas and Phil Evans for use of their CD spectropolarimeter; Dr. Karin Schnetz for vaccina virus topoisomerase I; Nicholas Krohn and Drs. Günter Feix, Gabor Igloi, and Marion Krieg for comments on the manuscript. We are grateful to Dr. Günter Feix for continuous support of this project.

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BI972620R