

The nonspecific DNA-binding and -bending proteins HMG1 and HMG2 promote the assembly of complex nucleoprotein structures

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The mammalian high mobility group proteins HMG1 and HMG2 are abundant, chromatin-associated proteins whose cellular function is not known. In this study we show that these proteins can substitute for the prokaryotic DNA-bending protein HU in promoting the assembly of the *Hin* invertasome, an intermediate structure in *Hin*-mediated site-specific DNA inversion. Formation of this complex requires the assembly of the *Hin* recombinase, the Fis protein, and three *cis*-acting DNA sites, necessitating the looping of intervening DNA segments. Invertasome assembly is strongly stimulated by HU or HMG proteins when one of these segments is shorter than 104 bp. By use of ligase-mediated circularization assays, we demonstrate that HMG1 and HMG2 can bend DNA extremely efficiently, forming circles as small as 66 bp, and even 59-bp circles at high HMG protein concentrations. In both invertasome assembly and circularization assays, substrates active in the presence of HMG1 contain one less helical turn of DNA compared with substrates active in the presence of HU protein. Analysis of different domains of HMG1 generated by partial proteolytic digestion indicate that DNA-binding domain B is sufficient for both bending and invertasome assembly. We suggest that an important biological function of HMG1 and HMG2 is to facilitate cooperative interactions between *cis*-acting proteins by promoting DNA flexibility. A general role for HMG1 and HMG2 in chromatin structure is also suggested by their ability to wrap DNA duplexes into highly compact forms.

[Key Words: HMG proteins; HU; DNA bending; site-specific recombination]

Communication between proteins bound to closely positioned sites on a DNA molecule, with the requisite looping of the intervening DNA segment, is a common feature of many biological reactions (for review, see Echols 1986; Ptashne 1986; Adhya 1989). When the intervening segment is very short, interactions between proteins bound at these sites may be inhibited as a result of the inherent stiffness of DNA. This inflexibility has been demonstrated by ligation assays showing that the minimal length of DNA that can form a circle is ~150 bp (Shore et al. 1981). DNA segments shorter than this cannot circularize without the aid of additional factors or sequences that serve to increase flexibility. Accessory proteins that fulfill this role may be important in regulating synergistic interactions between proteins bound to sites within complex regulatory elements.

Although little is currently known about such factors in eukaryotic systems, proteins have been described in prokaryotic systems that function specifically to promote cooperative interactions between *cis*-acting ele-

ments. The sequence-specific binding and bending protein, integration host factor (IHF), has been shown to promote interactions between high- and low-affinity integrase-binding sites in intasome assembly during phage λ site-specific recombination (Richet et al. 1986; Moitoso de Vargas et al. 1989; Kim et al. 1990), between DnaA proteins bound within the pSC101 replication origin (Stenzel et al. 1991), and between transcriptional activators and σ^{54} RNA polymerase (Gober and Shapiro 1990; Hoover et al. 1990). In the case of λ intasome assembly, an intrinsically bent DNA sequence or an unrelated DNA-bending protein can substitute for IHF binding, demonstrating that the ability of IHF to bend DNA is its principal function in this system (Goodman and Nash 1989; Goodman et al. 1992).

A related but nonspecific DNA-binding protein in prokaryotes, HU, strongly stimulates *Hin*-mediated site-specific recombination under certain conditions (Johnson et al. 1986; Haykinson and Johnson 1993). In this DNA inversion reaction, the *Hin* recombinase binds to sites located at either end of the invertible DNA segment on a supercoiled DNA molecule (Glasgow et al. 1989; Johnson 1991). The two *Hin*-bound *hix* sites synapse at

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the recombinational enhancer, which is bound at two sites by dimers of the Fis protein. This invertasome structure (Fig. 1) has been directly visualized by immunoelectron microscopy (Heichman and Johnson 1990). The formation of the invertasome is required to initiate DNA strand exchange, which is believed to occur by a rotation of Hin subunits after double-stranded cleavage and covalent attachment to the DNA at the center of the *hix* sites (Kanaar et al. 1990; Heichman et al. 1991). The *cis*-acting Hin recombinational enhancer can be assembled into the invertasome when positioned over 4 kb from a *hix* site. Invertasome assembly in reactions containing just the Hin and Fis proteins, however, is inefficient when <104 bp is present between the center of the closest *hix* site (*hixL1* in Fig. 1) and the center of the proximal Fis-binding site on the enhancer (Haykinson and Johnson 1993). In the presence of the HU protein, invertasome assembly occurs both *in vitro* and *in vivo* with intervening DNA segments as short as 60 bp, provided the *hix* and enhancer sites are in the correct helical phase with respect to each other. By binding within the short intervening segment, HU is believed to mediate sufficient bending of the DNA to enable the physical association of *hixL1* with the enhancer.

HU is an abundant and highly conserved protein in prokaryotes (for review, see Drlica and Rouvière-Yaniv 1987; Pettijohn 1988). In *Escherichia coli*, there are an estimated 30,000 heterodimers per cell, with each dimer comprised of two highly related subunits. In addition to its function in Hin-mediated DNA inversion, HU has been shown to play important roles in other specific reactions such as phage Mu transposition (Craigie et al. 1985), Tn10 transposition (Morisato and Kleckner 1987), and chromosomal DNA replication (Dixon and Kornberg 1984). A general role in mediating chromosome compaction in the bacterial nucleoid has also been implicated, although mutants deficient in the synthesis of both subunits remain viable (Rouvière-Yaniv et al. 1979; Wada et al. 1988; Huisman et al. 1989).

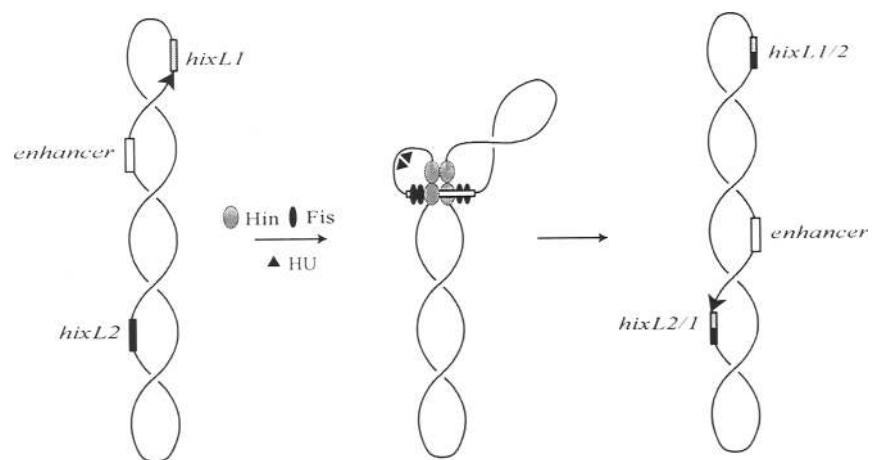
Although HU is the only protein from *E. coli* that can promote Hin invertasome assembly with short intervening segments, we found that an activity is also present in mammalian nuclear extracts that can efficiently substitute for HU (Haykinson and Johnson 1993). In this study, we have used the Hin inversion reaction as an assay for general eukaryotic factors that can facilitate cooperative interactions between *cis*-acting proteins. We show that the abundant chromatin-associated high mobility group (HMG) proteins 1 and 2, like HU, can promote invertasome assembly irrespective of DNA sequence. By ligase-mediated circularization of short DNA fragments, we demonstrate that HMG1 and HMG2 can bend DNA extremely efficiently, generating smaller DNA circles than is possible with HU. Finally, we examine the activity of isolated structural domains of HMG1 with respect to invertasome assembly and DNA bending.

Results

HMG proteins in HeLa nuclear extract can substitute for HU in invertasome assembly

In our initial search for eukaryotic proteins that could substitute for HU in the Hin inversion reaction, we used reaction conditions that result in invertasome assembly without strand exchange (Johnson and Bruist 1989). Incubation of a plasmid substrate with Hin and Fis in the absence of Mg²⁺ and the presence of ethylene glycol results in the accumulation of invertasome structures, as measured by double-strand cleavage by Hin within both recombination sites. We have shown previously that invertasome assembly on a plasmid containing 83 bp between *hixL1* and the enhancer is very inefficient unless HU is present (Haykinson and Johnson 1993). As shown in Figure 2A, a nuclear extract derived from HeLa cells efficiently substituted for HU in promoting Hin-mediated DNA cleavage, as evidenced by the release of invertible DNA segment from the vector. At a high protein concentration the activity is masked by strong topoi-

Figure 1. The Hin-mediated DNA inversion reaction. A schematic model is shown for the initial supercoiled substrate (*left*), the Hin invertasome (*center*), and the rearranged product (*right*). The recombination substrates used in this study contain the enhancer located between the Hin-binding sites (*hixL1* and *hixL2*), but much closer to *hixL1* (40–96 bp) than to *hixL2* (703 bp). The *hix* sites bound by Hin recombinase dimers (hatched spheres) are associated together at the enhancer, which is bound by two Fis dimers (solid ellipses). HU (solid triangles) or as shown in this paper, HMG1 and HMG2, facilitate this process when the separation between a *hixL* site and the enhancer is short (<100 bp). The precise locations and stoichiometry of proteins within this complex are not known. The Hin recombinase cleaves and exchanges the DNA strands, resulting in the inversion of the DNA segment between the *hix* sites.



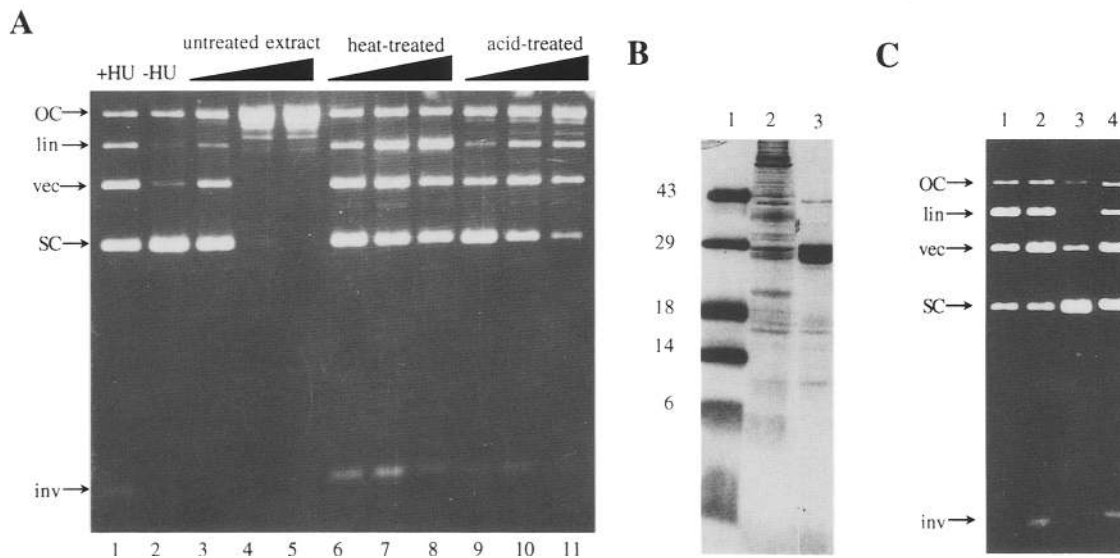


Figure 2. Activity of HeLa nuclear extract in the cleavage reaction. (A) Shown are Hin cleavage reactions using pMS551-83, which has 83 bp between *hixL1* and the enhancer. Reactions were performed with Hin, Fis, and either HU (lane 1; 100 ng), buffer only (lane 2), untreated HeLa nuclear extract (lanes 3–5; 0.1, 0.5, 2.5 μ g), nuclear extract heated at 80°C for 15 min (lanes 6–8; 0.1, 0.5, 2.5 μ g), or nuclear extract incubated for 30 min with 0.1 N HCl followed by neutralization with 0.1 N NaOH (lanes 9–11; 0.1, 0.5, 2.5 μ g). Arrows indicate the locations of the invertible segment (inv), vector (vec), and linear plasmid (lin) bands that are the products of Hin cleavage, along with the supercoiled (SC) and open circular forms (OC) of the substrate plasmid. (B) SDS-PAGE of HeLa nuclear extract and HeLa HMG proteins. Molecular weight standards (lane 1), 2 μ g of nuclear extract (lane 2), and 1.125 μ g of crude HMGs from the same extract (lane 3) were electrophoresed on a 15% SDS–polyacrylamide gel and stained with Coomassie blue. (C) Cleavage reactions with pMS551-83 performed in the presence of HeLa HMG proteins. The plasmid was incubated with Hin, Fis, and 225 ng of crude HMGs from the HeLa extract (lane 1), 200 ng of heat-treated nuclear extract (lane 2), buffer only (lane 3), or proteins from the ~29-kD doublet HMG band, which were excised, eluted, and renatured from an SDS–polyacrylamide gel (lane 4; see major bands in B lane 3).

somerase activities present in the extract, but strong stimulation is seen at the lowest concentration (lane 3). The HeLa activity was found to be resistant to heat inactivation (80°C for 15 min, lanes 6–8) or acid treatment (0.1 N HCl for 30 min, lanes 9–11) which largely inactivate the topoisomerase. The specific activity of the heat-treated nuclear extract was three to five times greater than the specific activity of heat-treated bacterial extracts as assayed by Hin-mediated cleavage (data not shown).

To identify the source of HU-like activity in the nuclear extract, heat-treated preparations were fractionated by gel filtration, ion-exchange, and reverse-phase chromatography and assayed using the Hin cleavage reaction. During the course of these experiments, the activity was always associated with a doublet of proteins migrating at ~29 kD on denaturing polyacrylamide gels (data not shown). Given these results, we reasoned that good candidates for the source of the activity in the HeLa extracts are a subset of the HMG proteins. HMG1 and HMG2, like HU, are relatively abundant, bind DNA without sequence specificity, and resist heat and acid treatment (Shooter et al. 1974; Goodwin et al. 1975; Johns 1982; Kuehl et al. 1984; Drlica and Rouvière-Yaniv 1987). HMG1 and HMG2 are ~25 kD in molecular mass but migrate at ~29 kD on SDS–polyacrylamide gels. We therefore isolated crude HMG proteins from the HeLa extract using a protocol involving perchloric acid extraction and acetone precipitation (Johns 1982) and found

that these were fully active in the cleavage reaction (Fig. 2B, C). The doublet of proteins migrating at ~29 kD were excised from an SDS–polyacrylamide gel, eluted, renatured after treatment with guanidine hydrochloride, and found to be still active in our assay (Fig. 2C, lane 4). This result strongly implies that human HMG1 and HMG2 can substitute for HU in the formation of the invertasome complex.

Purified bovine HMG1, HMG2, and HMG3 can substitute for HU in the inversion reaction

Mammalian HMG proteins are known to be almost completely conserved, with bovine HMG1 containing just one amino acid difference from its human counterpart (Kaplan and Duncan 1988; Wen et al. 1989). To obtain larger amounts of material for further characterization, pure HMG proteins were obtained from bovine thymus tissue. Crude HMG protein was extracted from chromatin using trichloroacetic acid (TCA) and acetone precipitations (Goodwin et al. 1973) and pure HMG1 and HMG2 were obtained by ion exchange chromatography on polybuffer exchange resin. A major degradation product of bovine HMG1, called HMG3, which lacks the acidic tail of HMG1 (see below; Johns 1982), was isolated by a combination of ion-exchange and reverse-phase chromatography. HMG14, a protein related to HMG1 and HMG2 in terms of solubility and chromatin association, but not by sequence (Bustin et al. 1990), was also

partially purified by similar methods as HMG3. The identities of all of these purified proteins were confirmed by amino acid analysis, and HMG1 and HMG2 were distinguished by amino-terminal amino acid sequencing (data not shown).

Purified HMG1, HMG2, HMG3 and HU were assayed for their ability to stimulate Hin-mediated inversion using a substrate containing 96 bp between the enhancer and *hixL1* site (Fig. 3A). HMG1 and HMG2 had very similar activities, and both seemed to be more efficient than HU in promoting inversion, especially at low protein concentrations where up to 2.5-fold higher inversion rates are obtained with the HMG proteins as compared with HU (Fig. 3A and data not shown). HMG3 was also active, although to a slightly lesser extent than HMG1 and HMG2, demonstrating that the acidic tail of HMG1 is not necessary for HU-like function. In Figure 3B, we compare the efficiency of HMG1 and HMG2 in the Hin cleavage assay with a substrate containing an 83-bp intervening segment. As discussed above, this assay measures only the ability of the HMG proteins to facilitate assembly of the invertasome without requiring that the DNA strands be exchanged and is more easily quantitated. The activities of HMG1 and HMG2 are indistinguishable in this assay, yielding a sevenfold stimulation in levels of cleaved DNA molecules. Determination of precise stoichiometry is not possible from these experiments, but the fact that the rate of invertasome assembly on 0.1 pmole of substrate DNA increases with <0.1 pmole of HMG protein suggests that only one or a few monomers of HMG1 or HMG2 per DNA substrate may be sufficient. These results are very similar to what we have reported previously for HU (Haykinson and Johnson 1993).

The ability of HMG1 to assemble invertasomes on substrates containing different lengths of intervening DNA was also measured. The results for substrates in which *hixL1* and the enhancer are in the optimal helical alignment with respect to each other are depicted in Figure 4A. We find that HMG1 is able to promote invertasome assembly with shorter lengths of DNA between the *cis*-acting sites than HU. HMG1 increases the rate of invertasome assembly with a 51-bp intervening segment approximately seven-fold, whereas no stimulation is obtained at this length in the presence of HU. The rate of invertasome assembly with a 63-bp intervening segment is ~50% greater with HMG1 than HU. Neither protein can promote invertasome assembly on a substrate containing a 40-bp intervening segment, and equivalent stimulation is seen at 73 bp or greater separation between *hixL1* and the enhancer.

The DNA substrates used in the cleavage and inversion reactions described above contain several A-tracts—short runs of A or T nucleotides sequentially on one strand—in the intervening segment between the *hixL1* and the enhancer. Because A-tracts are associated with intrinsically curved DNA (for review, see Travers and Klug 1990; Hagerman 1992), and some members of the HMG protein family—HMG1/Y and chicken HMG2a—are known to preferentially bind to AT-rich regions of DNA (Brown and Anderson 1986; Solomon et al. 1986; Russnak et al. 1988; Thanos and Maniatis 1992), we wanted to determine whether the A-tracts in our recombination substrates influence the function of HMG proteins in these assays. For this purpose we used a substrate whose intervening segment is 63% GC-rich and contains only two AA or TT dinucleotides (pMS551-97.1). As shown in Figure 4B, the rates of inversion ob-

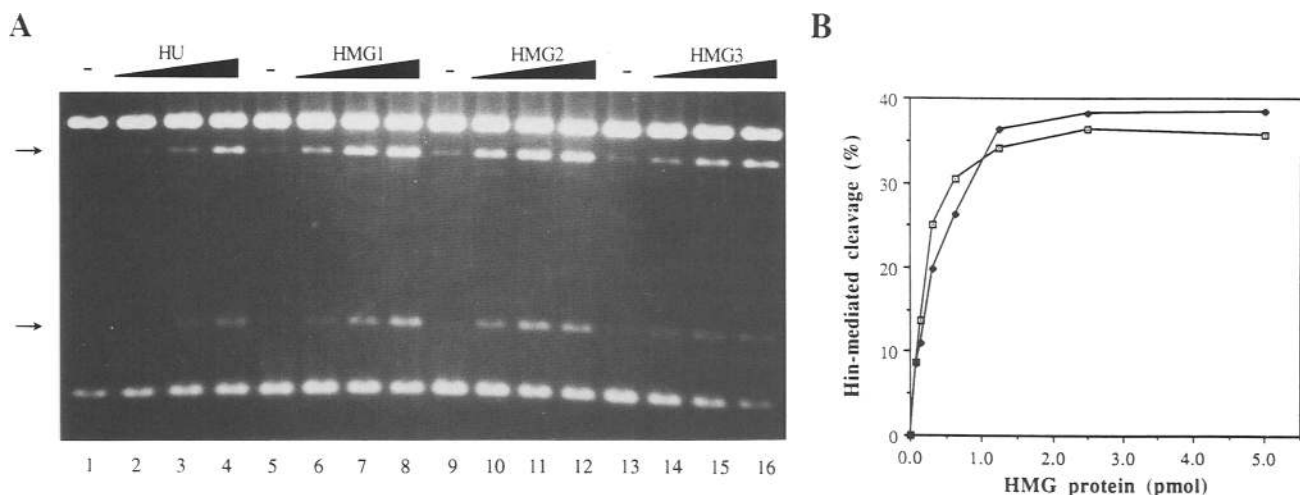


Figure 3. Activity of purified bovine HMG proteins in Hin-mediated recombination. (A) Inversion reactions with 0.1 pmole of pMS551-96 and purified HMG or HU proteins are shown. Inversion reactions were performed for 4 min with Hin, Fis, and buffer only (lanes 1, 5, 9, 13), HU (lanes 2–4), HMG1 (lanes 6–8), HMG2 (lanes 10–12), or HMG3 (lanes 14–16). Protein/DNA molar ratios for each protein used were 2 : 1, 10 : 1, and 50 : 1, respectively (note that 1 molecule of HU = 1 α/β dimer). Arrows point to bands indicative of inversion. (B) Cleavage reactions with 0.1 pmole of pMS551-83. Reactions were performed for 4 min in the presence of varying amounts of HMG1 (□) or HMG2 (◆) as indicated. The background reaction (~20%) determined for each substrate in the absence of accessory factor was subtracted from each point shown.

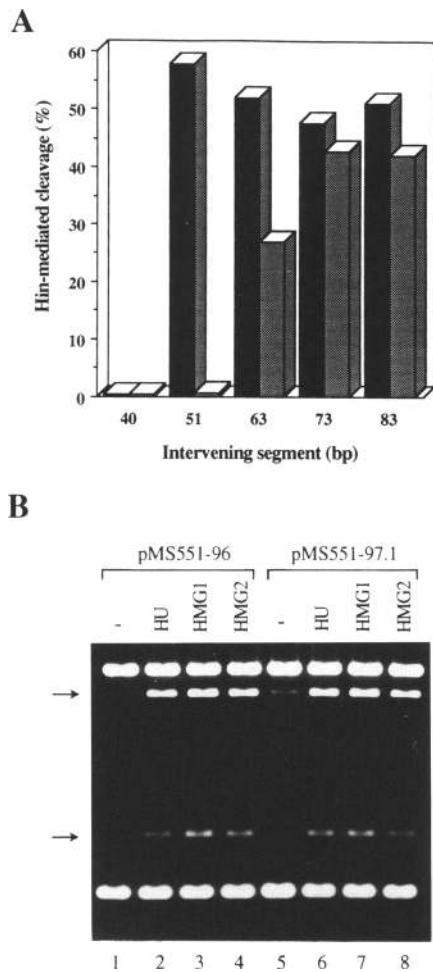


Figure 4. Activity of HU and HMG proteins on different recombination substrates. (A) HMG1 and HU stimulation of invertasome assembly with intervening segments of different lengths. pMS551-40, pMS551-51, pMS551-63, pMS551-73, and pMS551-83 were used in 4-min Hin cleavage reactions in the presence of 150 ng of HMG1 (solid bar), or 150 ng of HU (stippled bar). Background levels of invertasome assembly ranging from 10% to 20% cleavage obtained in the absence of accessory protein were subtracted from each point. (B) Inversion reactions with pMS551-96 vs. pMS551-97.1, which has an unrelated GC-rich sequence between *hixL1* and the enhancer. The plasmids were incubated for 4 min with Hin, Fis, and buffer only (lanes 1,5), 100 ng of HU (lanes 2,6), 100 ng of HMG1 (lanes 3,7), or 100 ng of HMG2 (lanes 4,8).

served with the substrates lacking the A-tracts were comparable to those containing the A-tracts, for HMG1, HMG2, and HU. Thus, in this assay, A-tracts do not seem to contribute significantly to the assembly functions of these proteins.

HMG1 and HMG2 bend DNA

As diagrammed in Figure 1, the invertasome is a topologically complex structure, involving precise arrangement of DNA strands as well as multiple protein-protein interactions. The HMG proteins could be promoting inver-

tasome assembly by binding to specific DNA structures, by directly stabilizing protein complexes, or by facilitating looping of the DNA between protein-binding sites. To investigate the latter possibility, we employed a ligation assay that has been used by others to demonstrate DNA-bending activity (Kotlarz et al. 1986; Dripps and Wartell 1987; Zahn and Blattner 1987; Hodges-Garcia et al. 1989; Schroth et al. 1989). In this assay, bending is measured by the circularization of a DNA substrate shorter than the average persistence length (~150 bp), which will not circularize in the absence of DNA-bending protein. The protein is incubated with ³²P-labeled DNA fragments at low concentrations, which favor intramolecular reactions, and then with T4 DNA ligase. Monomer DNA circles form during the reaction at a rate dependent on the concentration of protein and the ability of the protein to bend DNA. To differentiate between linear and circular molecules, the DNA is then incubated with exonuclease III, which digests linear but not circular ligation products.

HMG1, like HU (Hodges-Garcia et al. 1989), is very efficient in promoting the formation of monomer circles in the ligation assay (Fig. 5A). The initial 99-bp fragment is shown in lane 1 with no added protein, and in lane 2 with the addition of ligase—yielding dimer linear, dimer circular, and a trace of trimer linear species. The DNA in lane 3 was incubated with ligase and then with exonuclease III; thus, the linear ligation products are absent, whereas the dimer circle band remains. In the rest of the lanes, the DNA was incubated with increasing amounts of either HU (lanes 4–8) or HMG1 (lanes 9–13) before addition of ligase. Both proteins can efficiently promote formation of 99-bp monomer circles, which do not form in the absence of DNA-bending protein. Note that in all of the reactions with HU and HMG1, exonuclease III was used after ligation; thus, all of the bands seen are circular molecules. At this DNA concentration, monomer circles appear at a protein/DNA molar ratio of 4 : 1 (lanes 5,10), and levels appear to be maximal at a ratio of 8 : 1 with HMG1 (lane 11) and 16 : 1 with HU (lane 7). At higher concentrations of HU or HMG1 the levels of dimer circle decrease in favor of increased levels of monomer circle until the monomer species comprises 100% of the circular molecules.

As shown in Figure 5B, HMG2 and HMG3 are also active in the ligation assay. Levels of monomer circles seen with HMG2 are nearly identical to those seen with HMG1, whereas HMG3 is less active. As in the inversion assay, HMG14 shows no activity (not shown). Thus, the results of the ligation assays parallel the results of the cleavage and inversion assays and provide evidence that the function of HMG1 and HMG2 in promoting assembly of the invertasome complex may be to promote bending of the DNA substrate.

Distance dependence of DNA circularization mediated by HMG1 and HU

As mentioned above, the intrinsic torsional stiffness of short DNA fragments, as well as their helical repeat,

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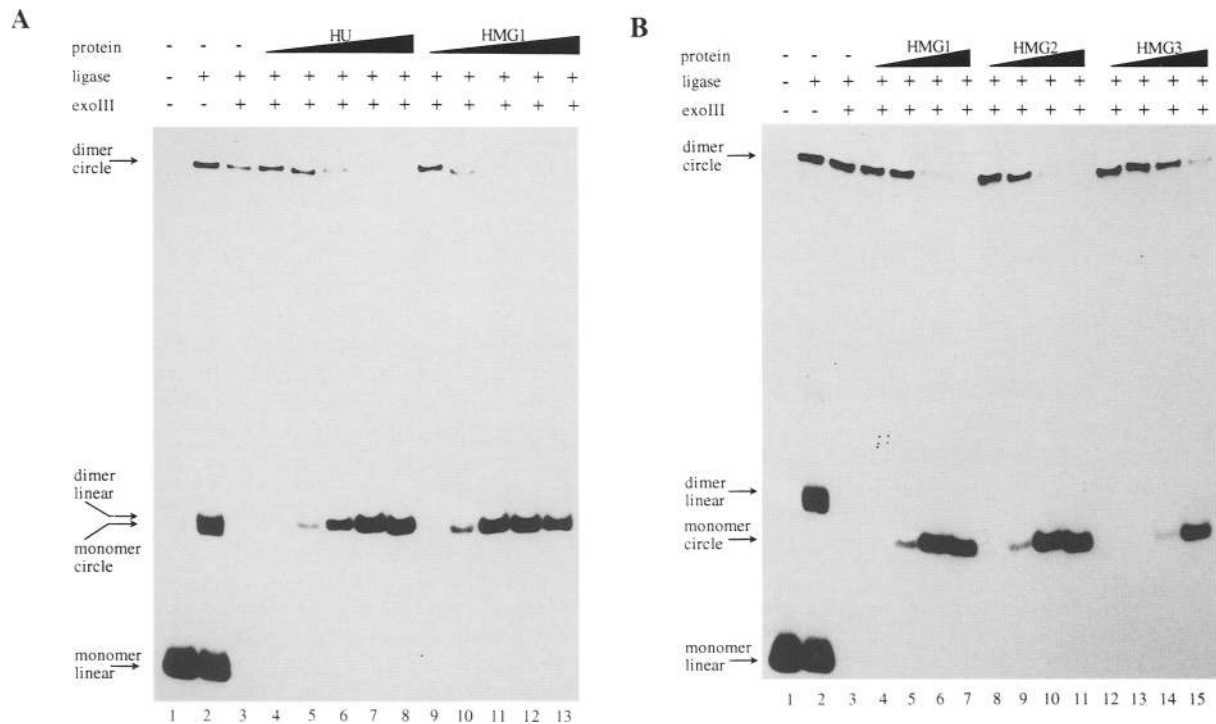


Figure 5. Circularization of 99 bp of DNA promoted by HU, HMG1, HMG2, and HMG3. (A) A 99-bp ³²P-labeled DNA fragment with *Eco*RI sticky ends was incubated with buffer only (lanes 1–3), HU (lanes 4–8), or HMG1 (lanes 9–13). T4 DNA ligase was added to reactions in lanes 2–13 and incubated at 30°C for 10 min. Reactions in lanes 3–13 were subsequently incubated with exonuclease III to remove linear ligation products. The reaction products were electrophoresed on a 7% polyacrylamide gel, which was then dried and autoradiographed. Protein/DNA molar ratios used were 2 : 1 (lanes 4,9), 4 : 1 (lanes 5,10), 8 : 1 (lanes 6,11), 16 : 1 (lanes 7,12), and 32 : 1 (lanes 8,13). (B) HMG1, HMG2, and HMG3 were used in ligation reactions as described in part A. Protein/DNA ratios used were 2 : 1 (lanes 4,8,12), 4 : 1 (lanes 5,9,13), 8 : 1 (lanes 6,10,14), and 16 : 1 (lanes 7,11,15). Note that the migration of the monomer circle species is variable in relation to the migration of the dimer linear species depending on the voltage used during electrophoresis.

impose constraints on DNA looping which may, in theory, be altered by DNA-bending proteins. To examine the effects of HMG1 and HMG2 on the efficiency of DNA circularization as a function of DNA length, we constructed a series of ligation substrates ranging from 59 to 99 bp, varying by one or a few base pairs at each step.

Efficient formation of monomer circles in reactions containing HMG1 (Fig. 6A) and HMG2 (not shown) was observed with fragments ranging from 66 to 99 bp. Levels of monomer circles varied in an oscillatory manner from 0 to 50% of total DNA, presumably in response to the strict requirement for an integral number of turns in a small DNA circle. At HMG:DNA concentrations of 32 : 1, a small number (4%) of 59-bp circles was obtained. Fourier analysis of the periodicity at HMG1/DNA ratios of 8 : 1, 16 : 1, and 32 : 1 gave an average repeat of 10.6–10.7 bp per turn, consistent with the average helical repeat of linear DNA of ~10.5 bp per turn determined by a variety of methods (Bellomy and Record 1990).

For comparison, a similar analysis was performed on HU. HU has been shown previously to promote circularization of a 99-bp fragment (Hodges-Garcia et al. 1989). We find that HU promotes circularization of fragments as small as 78 bp, and the pattern of activity also seems

to reflect the helical repeat of linear DNA (Fig. 6A). Unlike HMG1 and HMG2, however, HU was unable to circularize fragments shorter than 75 bp.

The various ligation fragments used in the ligation assays were generated from a set of DNA substrates that contain several A-tracts. To determine the effects of these sequences on HMG1-mediated DNA circularization, we also performed ligation assays on another set of DNA fragments. These substrates have a single A-tract located near one end of the molecule, but this would not be predicted to have a significant effect on either the overall intrinsic curvature of the DNA or on the protein-induced site-specific bending of the molecules (Wu and Crothers 1984). These fragments, ranging from 60 to 76 bp, were used in ligation assays with HMG1, and the results were comparable to the original substrates in the same size range (Fig. 6B). These substrates are efficiently circularized by similar amounts of HMG1, and the same general phasing pattern is retained.

Domain B of HMG1 is sufficient for bending activity

Protease digestion experiments, secondary structure predictions, and DNA-binding studies indicate that HMG1 and HMG2 have three major structural domains (Reeck

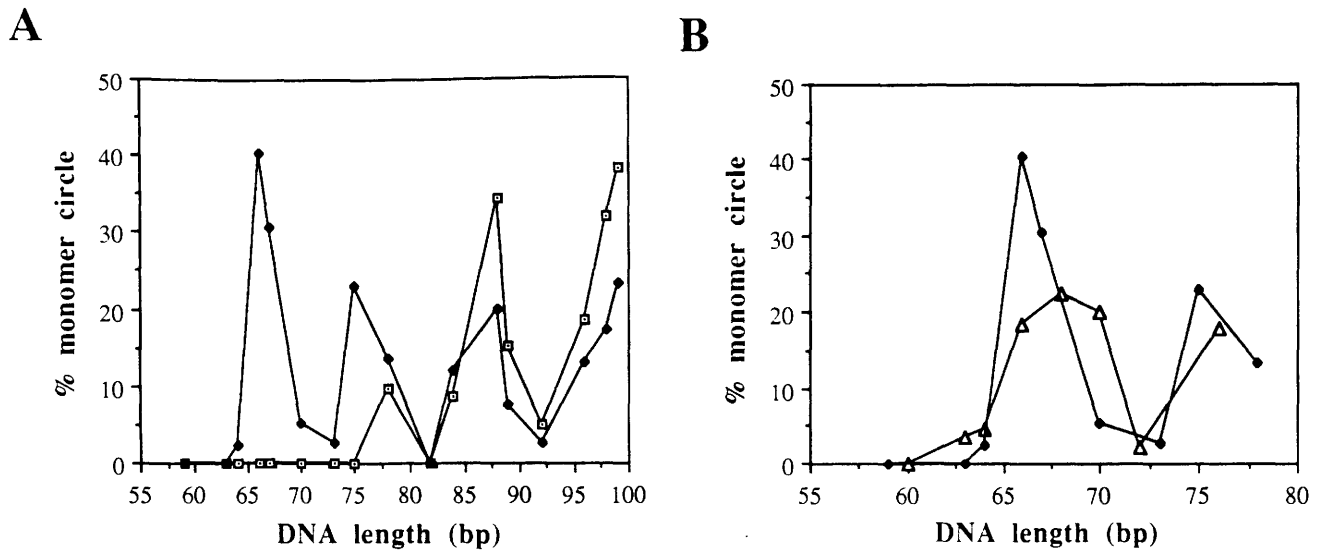


Figure 6. Circularization of 59- to 99-bp DNA fragments promoted by HMG1 and HU. (A) Circularization of labeled DNA substrates ranging from 59 to 99 bp in the presence of HMG1 (\blacklozenge) and HU (\square) is shown using a protein/DNA ratio of 16 : 1. Ligation reactions were done as described in Fig. 5A, and each autoradiograph was scanned quantitatively. Percent (%) monomer circle refers to the ratio of the integrated density of the monomer circle band divided by the integrated density of the total input fragment electrophoresed in parallel. (B) Circularization of 59- to 76-bp labeled DNA substrates of different sequence in the presence of HMG1 is shown. Ligation reactions were performed at a HMG1/DNA ratio of 16 : 1 on DNA fragments lacking centrally located A-tracts (\triangle), and the results were plotted together with the data from the same range of fragment lengths from A (\blacklozenge).

et al. 1982; Carballo et al. 1983, 1984; Bianchi et al. 1992). The DNA-binding portions of the proteins are composed of two relatively basic regions, termed domains A and B, which are $\sim 47\%$ similar but only 27% identical to each other in amino acid sequence. The carboxy-terminal portions of the proteins contain the third, extremely acidic, domain. As diagramed in Figure 7, when HMG1 or HMG2 is incubated with low concentrations of V8 protease for a short time, intact protein fragments can be recovered that correspond roughly to the structural domains. Isolated V3 (approximately domain A) and V2 (approximately domain B) have high α -helical content and bind duplex DNA, whereas V1 (approximately domain B plus acidic tail) is comparatively unstructured and does not bind DNA (Carballo et al. 1983, 1984).

Figure 8A shows the results of inversion assays with isolated V2 and V3 peptides. The mix of protease digestion products, containing no intact HMG1 protein, was active in stimulating Hin-mediated DNA inversion (lane 3). Of the isolated domains, only V2 (domain B) showed activity, although significantly less than that of intact HMG1. Similarly, when the digestion products were used in the ligation assay, only V2 (domain B) was active (Fig. 8B). Even though V1 contains domain B, it has been shown to be quite different from V2 alone, having very little secondary structure as measured by spectroscopic techniques (Carballo et al. 1984). This effect has been ascribed to destabilizing effects of the acidic tail on domain B when separated from domain A. V1, like V3, was not active in DNA bending (Fig. 8B) or in inversion stimulation (data not shown).

To determine whether our preparation of V3 yielded an intact peptide, we examined its ability to bind linear and branched DNA structures by use of filter-binding assays. Bianchi and co-workers have demonstrated that both domains A and B have a high affinity for cruciform DNA (Bianchi et al. 1992). Filter-binding assays were

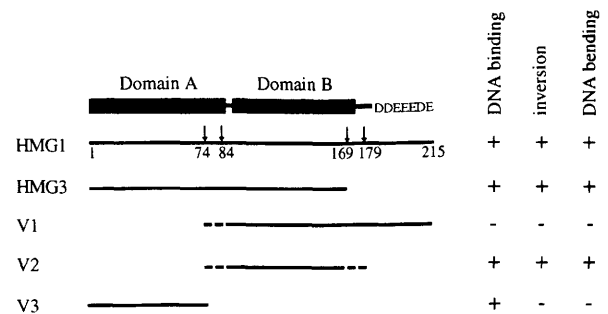


Figure 7. Domain structure of HMG1 and summary of the activities of HMG1 and its proteolytic derivatives. The approximate locations of domain A (amino acids 1–89), domain B (amino acids 90–174), and the acidic tail (amino acids 186–215) of HMG1 are shown. HMG3 is a natural degradation product of bovine HMG1, which is missing the carboxy-terminal tail (Johns 1982). Partial proteolysis of HMG1 with V8 protease yields three major products: V1 (domain B and the acidic tail), V2 (approximately domain B), and V3 (approximately domain A). The locations of the amino- and carboxy-terminal positions of the peptides are based on our amino acid composition and mass spectroscopy data and the analysis of Carballo et al. (1983). The presence of residues 74–84 and 169–179 in V2 is variable because of multiple protease cutting sites.

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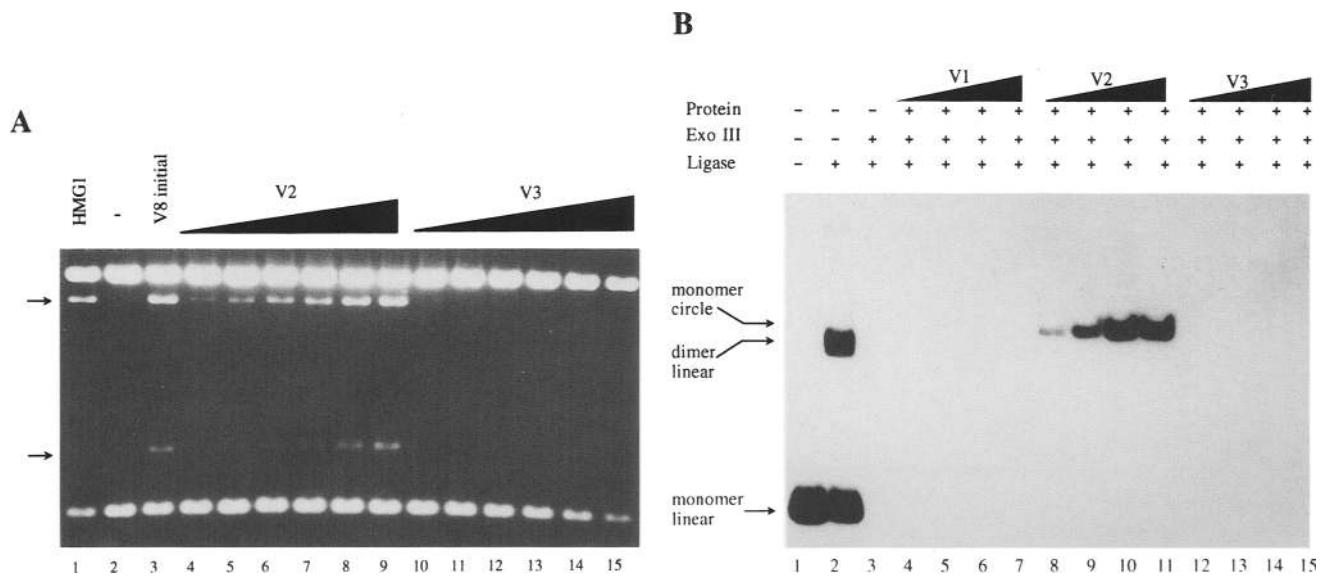


Figure 8. Activity of isolated V8 digestion products of HMG1. (A) V2 (approximately domain B) and V3 (approximately domain A) were used in the *Hin* inversion reaction as described in Fig. 3A. Intact HMG1 (100 ng) was added in lane 1, buffer only in lane 2, and a mix of V8 digestion products before separation (V8 initial, 1 μ g total) in lane 3. Amounts of isolated domains added to reactions ranged from 30 ng to 1 μ g in two-fold increments as denoted. (B) Ligation assays were performed on 99-bp labeled DNA fragments in the presence of V1 (approximately domain B and carboxy-terminal acidic tail), V2 (approximately domain B), and V3 (approximately domain A) as described in Fig. 5A. Protein/DNA ratios for each peptide were 4 : 1 (lanes 4,8,12), 8 : 1 (lanes 5,9,13), 16 : 1 (lanes 6,10,14), and 32 : 1 (lanes 7,11,15). The calculated protein/DNA ratios for V2 and V3 assume that each is acting as a homodimer (Bianchi et al. 1992).

performed with HMG1, V2, and V3 in the presence of 32 P-labeled Holliday junction DNA or linear DNA. Significant levels of binding to both Holliday structures and linear DNA were seen with each of the proteins tested (data not shown). We did observe, however, that our V3 preparation gave a less stable complex in mobility shift assays with Holliday junction DNA compared with V2, HMG1, and HU, although no significant difference in cruciform DNA binding between HMG1 domains was noted by Bianchi et al. (1992). Interestingly, HU gave the most stable complexes with Holliday junction DNA in mobility shift assays compared with HMG1, V2 and V3, consistent with a recent report that HU binds specifically to four-way DNA junctions (Pontiggia et al. 1993). It is possible that the physical differences between cloned domains and protease-generated domains are responsible for the apparently weaker binding of our V3 preparation in the mobility shift assays (see Discussion). Alternatively, the structural differences between cruciforms and the more dynamic Holliday junctions may account for this variance.

Discussion

In this study we demonstrate that the chromatin-associated mammalian proteins HMG1 and HMG2 exhibit activity greater than that of HU in stimulating DNA recombination in the *Hin*-mediated site-specific inversion reaction. Higher inversion rates are obtained with HMG1 and HMG2 as compared with HU, particularly

when limiting accessory protein is present. When invertasome assembly was directly measured in the *Hin* cleavage assay, HMG1 and HMG2 were found to increase rates of invertasome assembly up to 10-fold, depending on the DNA substrate assayed. In addition, the HMG proteins were found to promote invertasome assembly with shorter intervening segments between the enhancer and *hix* sites than was possible with HU. For example, whereas HU can promote moderate levels of invertasome assembly on DNA substrates with intervening segments of 63 bp (Haykinson and Johnson 1993), HMG1 and HMG2 can efficiently promote invertasome assembly on substrates containing just 51 bp between *hix* and the enhancer. Like HU, only a few molecules of HMG1 or HMG2 per supercoiled substrate are needed for stimulation.

The HMG proteins are a family of chromatin-binding proteins classified according to solubility, charge, and molecular weight (Bustin et al. 1990). HMG1 and HMG2 are abundant members of the group, present in a ratio of 0.2–0.3 molecules each per nucleosome as measured in several rat tissues (Kuehl et al. 1984). They bind double- and single-stranded DNA with no apparent sequence specificity, although they bind with markedly higher affinity to cruciform structures (Wright and Dixon 1988; Bianchi et al. 1989) and *cis*-platin-modified DNA (Pil and Lippard 1992). The exact functions of HMG1 and HMG2 are not yet known, although stimulatory effects of HMG1 and HMG2 on transcription (Stoute and Marzluff 1982; Tremethick and Molloy 1986,1988; Singh and

Dixon 1990), DNA replication (Alexandrova et al. 1984; Alexandrova and Beltchev 1988), and nucleosome assembly (Bonne-Andrea et al. 1984) have been reported. Our results with the *Hin* site-specific recombination reaction suggest that one specific function of HMG1 and HMG2 is to promote assembly of higher order nucleoprotein complexes, a feature that may be common to each of the processes cited above.

Although HMG proteins are not related to HU in sequence, it appears that they may play a similar functional role. HU is known to stimulate assembly of prepriming replication complexes *in vitro* (Baker et al. 1986; Skarstad et al. 1990), as well as the assembly of recombination intermediates in phage Mu transposition (Lavoie and Chaconas 1990; Baker and Mizuuchi 1992) and *Hin*-mediated inversion (Johnson et al. 1986; Haykinson and Johnson 1993). The known properties of HU, HMG1, and HMG2 are distinct from their sequence-specific counterparts: IHF in bacteria and LEF1 (the clearest example) in mammals. Both IHF and LEF-1 bind and bend DNA at specific recognition sites (Robertson and Nash 1988; Thompson and Landy 1988; Yang and Nash 1989; Giese et al. 1991, 1992). The binding of LEF-1 or the presence of intrinsically curved DNA can functionally substitute for IHF in the phage λ intasome reaction (Goodman and Nash 1989; Giese et al. 1992; Goodman et al. 1992). Thus, at least in this system, IHF and LEF-1 appear to play structural roles in facilitating productive contacts between proteins bound to adjacent DNA sites. It is proposed that LEF-1 also fulfills a primarily structural role when bound in the TCR α enhancer (Giese et al. 1992). HU, HMG1, and HMG2 seem to perform a similar function, but without the restriction of binding to a specific DNA recognition sequence.

DNA bending mediated by HMG1 and HMG2

We find that HMG1 and HMG2 have extraordinary DNA-bending activity, as demonstrated by their ability to promote circularization of very short DNA fragments. Enhancement of the ligation frequency of two ends of a short DNA fragment has been used previously to demonstrate DNA bending by HU on fragments ranging from 99 to 126 bp (Hodges-Garcia et al. 1989). We demonstrate in this work that HMG1 and HMG2, as well as HU, could promote ligation of fragments ≥ 78 bp in length. In contrast with HU, however, DNA circles of 66–75 bp could be formed efficiently in the presence of HMG1. Even circles as small as 59 bp were formed, albeit inefficiently. To our knowledge, these are the smallest DNA circles yet described. The enhanced ability of the HMG proteins to bend DNA relative to HU correlates with the ability of HMG1 to promote invertasome assembly on substrates containing 10 bp less DNA between recombination sites than is possible with HU.

The degree of DNA bending promoted by HMG1 and HMG2 in the ligation assays is even greater than that observed for DNA wound around a histone octamer. Within a nucleosome, 146 bp of DNA is wrapped ~ 1.8

times around a histone core with a diameter of ~ 86 Å (Richmond et al. 1984). The average diameter of a 66-bp circle (readily formed in the presence of HMGs) is only ~ 71 Å, indicating much greater bending of the DNA helix associated with HMG complexes than is present in a nucleosome.

Although HMG1 and HMG2 are dramatically bending DNA, we find no evidence for a significant change in the helical repeat. The periodicity of circularization of different length DNA fragments in the presence of HMG1 as calculated by Fourier analysis was 10.6–10.7 bp per turn, which is comparable with measurements of 10.45–10.6 bp per turn helical repeat on linear DNA *in vitro* (Bellomy and Record 1990). This conclusion is consistent with several observations that HMG1 and HMG2 are not unwinding or destabilizing the DNA helix (Shastri et al. 1982; Butler et al. 1985; Kohlstaedt et al. 1987), but is in contrast to other reports (Javaherian et al. 1979; Yoshida 1987; Sheflin and Spaulding 1989). It is possible, however, that a change in twist exerted by a single protein on the DNA molecule may not be detected in this assay. The periodicity of HU-promoted circularization was similar to that observed for HMG1, although sufficient data were not available to calculate a statistically significant helical repeat for HU. By measuring the efficiency of invertasome assembly, we also did not find that HU or HMG1 was altering the helical repeat of supercoiled DNA (Haykinson and Johnson 1993; data not shown). A significant preference of HMG1 or HMG2 activity on AT-rich regions was also not observed by either *Hin*-mediated inversion or ligation assays, in contrast to a report on chicken HMG2a (Brown and Anderson 1986) and another subgroup of HMG proteins, HMG1/Y, which bind preferentially to runs of five or more A or T nucleotides (Solomon et al. 1986; Russnak et al. 1988; Thanos and Maniatis 1992).

Activities of isolated polypeptides from HMG1

Previous work has established that HMG1 and HMG2 have three major domains (see Fig. 7): the amino-terminal and central regions (domains A and B), which have internal homology and can bind DNA independently (Carballo et al. 1983, 1984; Bianchi et al. 1992), and the carboxy-terminal acidic tail, which can interact with histone H1 *in vitro* (Carballo et al. 1983). HMG3, a degradation product of HMG1 missing the acidic tail, is active in promoting invertasome assembly as well as DNA bending, demonstrating that the carboxy-terminal region is not required for these functions. Analysis of V8-generated peptides of HMG1 indicate that domain B (peptide V2) alone is sufficient for promoting DNA bending and invertasome assembly. The peptide representing domain A (V3) was not capable of promoting invertasome assembly or circularization of short DNA fragments.

The differences in DNA bending and invertasome assembly activities between the V2 and V3 peptides could be indicative of functional differences between the two DNA-binding domains. On the other hand, these differences may reflect the fact that the V3 peptide does not

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contain the entire length of domain A, because we estimate that ~10 amino acids of domain A that include a conserved tyrosine residue in the degenerate consensus sequence called the HMG box (see below) are missing in the V3 peptide (see Materials and methods). This residue (Y77) is predicted to be in the conserved face of helix III, by analogy to the recently determined nuclear magnetic resonance (NMR) structure of HMG1 domain B (Weir et al. 1993). Bianchi et al. (1992) have expressed recombinant forms of the two domains and find that both are active in cruciform binding, as measured by gel electrophoresis assays. We show that whereas the V2 and V3 peptides are both active in binding linear or Holliday junction DNA in filter binding assays, the V3 peptide displayed markedly less stable binding to Holliday junctions in gel retardation assays. Thus, V3 is not functionally and may not be physically equivalent to domain A.

Although the ability of domain B (as well as intact HMG1 and HMG2) to mediate invertasome assembly is correlated most directly with its bending properties, we cannot rule out the possibility that the HMG proteins are functioning in the inversion reaction by stabilizing a particular DNA structure, such as the plectonemic branch, that is present in the invertasome. The cruciform binding activity demonstrated by the recombinant forms of each domain could be an expression of the same activity that we have measured in the invertasome assembly and ligation assays. An alternative model of HMG protein interactions involving direct protein-protein contacts between HMG proteins and invertasome components is unlikely because of the heterologous combination of the eukaryotic accessory factors and the prokaryotic recombination system.

Biological roles of HMG1 and HMG2

HMG proteins 1 and 2 are the founding members of a family of proteins that contain HMG boxes—regions that display sequence similarity to the 85-amino-acid DNA-binding domains of HMG1 and HMG2 (see, e.g., Jantzen et al. 1990; Diffley and Stillman 1991; Ferrari et al. 1992; Fisher et al. 1992; Giese et al. 1992). Unlike HMG1 and HMG2, however, many of these proteins bind DNA at specific recognition sites. Four of the known sequence-specific HMG box proteins: LEF-1, SRY, *stell*, mtTF1, and its yeast counterpart ABF2, have been shown to bend DNA (Diffley and Stillman 1992; Ferrari et al. 1992; Fisher et al. 1992; Giese et al. 1992). Thus, HMG1 and HMG2 could be the prototypes for a wide variety of specific DNA-binding proteins whose activity may be affected by their ability to modulate DNA structure (see also Lilley 1992).

HMG1 and HMG2 could function in a variety of eukaryotic transcription, replication, or recombination reactions by promoting cooperative interactions of *cis*-acting DNA-binding proteins. For example, they may facilitate contacts between different proteins bound within or between complex promoter and enhancer elements. In addition to their ability to function in the *Hin* recombination reaction, the generality of their role in nucleopro-

tein complex assembly is illustrated by their ability to function in other well-characterized prokaryotic transcription and recombination reactions involving multiple *cis*-acting sites. HMG1 and HMG2 can substitute for IHF in stimulating activator-dependent transcription of the *nifHDK* promoter by σ^{54} holoenzyme (D. Berger and S. Kustu, pers. comm.) and at the attachment sites in the prophage excision reaction of phage λ (A. Segall, S. Goodman, and H. Nash, pers. comm.). In addition, HMG1 and HMG2 can replace HU in the assembly of a phage Mu transposition complex (B. Lavoie and G. Chaconas, pers. comm.) and can substitute for IHF and HU in certain aspects of the *Tn10* transposition reaction (R. Chalmers and N. Kleckner, pers. comm.).

The ability of HMG1 and HMG2 to wrap DNA to such a remarkable degree also suggests potential roles for these proteins in chromatin condensation and packaging. The relative instability of HMG1- and HMG2-DNA complexes, as compared with nucleosome-wrapped DNA, implies that structural transitions mediated by HMG1 and HMG2 may be quite dynamic. This characteristic could be particularly important in the many processes that involve transient changes in chromatin structure.

Materials and methods

Recombination and ligation substrates

The plasmids used as substrates in the recombination assays are all described by Haykinson and Johnson (1993). They are derivatives of pMS551, which contains *hixL1* and the enhancer substituted between the *EcoRI* and *HindIII* sites and *hixL2* inserted at the *SalI* site of pBR322, as schematically shown in Figure 1. The plasmids are named by the number of base pairs between the center of *hixL1* and the center of the proximal *Fis*-binding site in the enhancer. Thus, pMS551-83 and pMS551-96 contain 83- and 96-bp intervening segments, respectively.

Ligation substrates were made using PCR on the above set of recombination plasmids using primers that incorporated *EcoRI* sites at the ends. Fragments were internally labeled with [α - 32 P]dATP, purified by polyacrylamide gel electrophoresis, and digested with *EcoRI*. DNA concentrations were estimated by comparing intensities of ethidium bromide staining of an aliquot that was electrophoresed in parallel with known amounts of DNA standards of similar size. Fragment preparations were used within a week.

Proteins

Hin, *Fis*, and HU were purified as described (Haykinson and Johnson 1993). HeLa nuclear extracts (a gift from M. Carey, University of California School of Medicine, Los Angeles) were prepared from a 0.15 M KCl wash of HeLa nuclei as described by Dignam et al. (1983). HMG proteins were selectively extracted from HeLa nuclei by the method described in Johns (1982). Renaturation of HMG1 and HMG2 from an SDS-polyacrylamide gel was performed essentially as described in Briggs et al. (1986) except that the acetone pellet was resuspended in 5 μ l of the guanidine-HCl solution for 15 min at room temperature followed by addition of 245 μ l of renaturation buffer. This solution (25 μ l) was used in the cleavage assay shown in Figure 2C.

The large-scale extraction of HMG proteins from calf thymus

was essentially as described by Goodwin et al. (1973), except that the 0.35 M NaCl extract was fractionated into 2% and 10% TCA precipitates, the 10% precipitate being the crude HMG proteins. HMG1 and HMG2 were separated on polybuffer exchange resin PBE94 (Pharmacia) as described by Adachi et al. (1990). To purify other HMG proteins not retained on the polybuffer column, the supernatant of the 10% TCA solution was made 22% in TCA, and the resulting precipitate was washed in acetone, dried, and resuspended in buffer A (10 mM Tris-HCl at pH 7.8, 1 mM DTT). Approximately 20 mg of protein from this preparation was applied to an FPLC Mono-S column (Pharmacia) and eluted with a continuous gradient from 50 mM to 1 M NaCl in buffer A. Fractions corresponding to 0.375–0.4 M NaCl that were active in the Hin cleavage assay were applied to a HR5/10 reverse-phase FPLC column (Pharmacia) in 0.1% trifluoroacetic acid (TFA) and eluted in a continuous gradient from 0 to 100% acetonitrile in 0.1% TFA. Fractions containing HMG3 and HMG14 eluted at ~30% and ~10% acetonitrile, respectively. Amino acid analysis was performed on aliquots of the HMG1, HMG2, HMG3, and HMG14 preparations to confirm their identity. Amino acid sequence analysis of the amino-terminal 18 residues of HMG1 and HMG2 unambiguously identified lysine and asparagine at residue 7 of HMG1 and HMG2, respectively, which enabled these two related proteins to be distinguished. All proteins were quantified by Bradford (Bio-Rad) assays with BSA as the standard. The amounts of HMG1 and HMG2 estimated by the Bradford assay were within 5–10% of the amounts determined by quantitative amino acid analysis.

V8 protease digestions of HMG1 were performed essentially as described by Carballo et al. (1983). The resulting peptides were isolated by chromatography on PBE94, which retains V1, followed by chromatography on an HR5/10 FPLC column, as described above, which separates V2 from V3. The boundaries of V1, V2, V3, and HMG3 were estimated by amino acid composition analysis performed on the preparations used in this study and from the data of Carballo et al. (1983) for V1, V2, and V3 and Johns (1982) for HMG3. The molecular mass of V3 was also measured by electrospray mass spectrometry to be 8532 ± 0.2 daltons, which best corresponds with the predicted molecular mass of 8536 daltons for amino acids 2–74. (The amino-terminal methionine was absent in the amino-terminal sequence analysis of HMG1.) These data suggested that the carboxy-terminal boundary of V3 is the V8 recognition site at amino acid 74 and that the boundaries of the predominant V2 peptide are the V8 cleavage sites at 74 and 169 or possibly at 85 and 179. Our amino acid composition of HMG3 matched very well with a polypeptide derived from HMG1 that extends from amino acid 2 to 168.

Recombination assays

In vitro inversion and cleavage assays were performed as described previously (Haykinson and Johnson 1993). Incubations were typically for 3–4 min to measure reaction rates.

Ligation assays

Ligations were performed in a volume of 10 μ l containing 50 mM HEPES (pH 7.5), 50 mM potassium glutamate, 10 mM magnesium acetate, and 1 mM ATP in the presence of 2 ng of labeled DNA fragment and HU or HMG protein as indicated. After 10 min at 30°C, 0.6 Weiss units of T4 DNA ligase (New England Biolabs) was added and incubated for 10 min followed by inactivation of the ligase by shifting to 65°C for 15 min. Some reactions were then incubated with 1 unit exonuclease III (Pharmacia) for 45 min at 37°C prior to incubation with 0.5% SDS

and proteinase K (200 μ g/ml) for 20 min. The samples were electrophoresed in a 7% polyacrylamide (60 : 1 acrylamide : bis) gel containing 10% glycerol in Tris–borate–EDTA buffer at 0.3–0.5 V/cm for ~12 hrs. The electrophoretic migration of circular DNA molecules is slower relative to the linear species at lower currents. The identities of the bands seen in the ligation assays were confirmed, in part, by the method described in Dripps and Wartell (1987). Band intensities from autoradiographs were quantitated by densitometry, and Fourier transformation of the ligation data were performed as described in Haykinson and Johnson (1993).

DNA-binding assays

Filter binding assays were performed in the ligation buffer described above minus ATP by incubating different amounts of proteins with either 1 pmole of ³²P-labeled Holliday structures or 0.1–0.5 pmole ³²P-labeled 240-bp linear DNA in a 20- μ l reaction volume. After 10 min at 23°C, the solution was applied to a 0.1- μ m nitrocellulose filter (Schleicher & Schuell) and washed in the same buffer. Protein/DNA-binding reactions for the mobility retardation assays were performed in the same buffer, and reaction products were electrophoresed in a 5% polyacrylamide gel at either 4°C or 23°C as described by Osuna et al. (1991). ³²P-Labeled Holliday structures, formed by the annealing of 70- to 85-bp oligonucleotides representing the FLP recombination site, were generously provided to us by J. Dixon and P. Sadowski (University of Toronto, Canada).

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The nonspecific DNA-binding and -bending proteins HMG1 and HMG2 promote the assembly of complex nucleoprotein structures.

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