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Interactions of recombinant HMGB proteins with branched RNA substrates

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ABSTRACT

The high mobility group protein HMGB1 is a highly abundant chromosomal protein known to interact preferentially with DNA that is branched, bent or otherwise structurally altered. Biologically the protein is thought to facilitate promoter attachment by transcription factors. Recently, however, HMGB1 has been shown to have biological roles beyond that of an architectural DNA-binding protein. Here we investigate the binding interactions of recombinant HMGB1 proteins with two branched RNA's *E. coli* 5S rRNA and the group I intron ribozyme from *Azoarcus* pre-tRNA^{Ile}. Using competitive electrophoretic mobility and circular dichroism binding assays, we show that HMGB proteins bind both substrates with high affinity. We also report that a recombinant rat HMGB protein, rHMGB1b, inhibits RNA cleavage by the ribozyme. These results raise the possibility that HMGB proteins possess structure dependent RNA binding activity and can modulate RNA processing as well as transcription.

High mobility group (HMG) proteins are among the most abundant non-histone chromosomal proteins, present at levels ca. 10^6 per nucleus. HMGB1 is a canonical HMG protein that binds strongly to cruciform or bent DNA and has been implicated in a variety of nuclear functions such as transcription, DNA repair, recombination and chromatin fiber assembly [1,2]. Due to its high copy number in mammalian cells, HMGB1 has been considered a nuclear housekeeping product. However, HMGB1 has been shown to be a key component of alternate cellular pathways that mediate neuronal differentiation, stem cell recruitment and innate immunity; current research focuses on defining the role of HMGB1 as a pro-inflammatory cytokine [3].

HMGB1 has a tripartite structure, consisting of two HMG box subunits (A and B) together with an acidic C-terminus (Fig. 1A). Each box subunit possesses a characteristic global fold of three α-helices arranged in an L-shaped structure [2]. Each HMG box is composed of a conserved sequence of ~80 amino acids that are rich in basic, aromatic and proline residues typically present in DNA-binding proteins [1]. Individual boxes of HMGB1 bind weakly to duplex DNA but strongly to bent and distorted DNA structures such as four-way junctions (4WJs), super-coiled and cisplatin modified DNA [4,5]. We have shown previously that recombinant HMG proteins containing tandem HMG boxes, arranged in either an AB or BA alignment, bind 4WJ DNA with higher affinity than individual HMG boxes [6].

While HMGB1 is a well-established DNA-binding protein, its role in alternate cellular pathways is not fully characterized, raising the possibility that HMG proteins may interact with a wider variety of nucleic acid substrates. For example the canonical HMG protein, HMGD, binds strongly to double-stranded regions of two HIV-1 regulatory RNA structures: the transactivation response region (TAR) and the Rev binding protein element (RBE) [7]. Other DNA-binding proteins have been reported to interact with RNA as well. The tumor suppressor p53 has also been found to bind RNA in a sequence-nonspecific manner that promotes RNA–RNA annealing [8].

To explore the role of RNA binding in the potential functions of HMGB1, we have investigated the nonspecific binding interactions of recombinant HMGB1 proteins with unfractionated *E. coli* tRNA. We found no evidence for interaction in the case of tRNA, so we turned next to longer branched RNA species, including 5S, 16S and 23S *E. coli* rRNA's. Experiments using labeled junction DNA as a probe revealed competition with each of these RNA's. Fig. 2 shows the data for 5S rRNA. Electrophoretic mobility shift (EMSA) and circular dichroism (CD) binding assays indicate that HMGB proteins, HMGB1b and HMGB1ab, bind 5S rRNA with high affinity. To detect functional consequences of HMG interactions with branched RNA, we tested the effect of HMG binding on the RNA splicing activity of the group I intron ribozyme from *Azoarcus* pre-tRNA^{Ile} (L-3). Binding of rHMGB1b to the ribozyme significantly reduces its RNA cleavage activity. Taken together, our data suggest that HMGB1 interacts with branched RNA species and thus may play a role in RNA processing.

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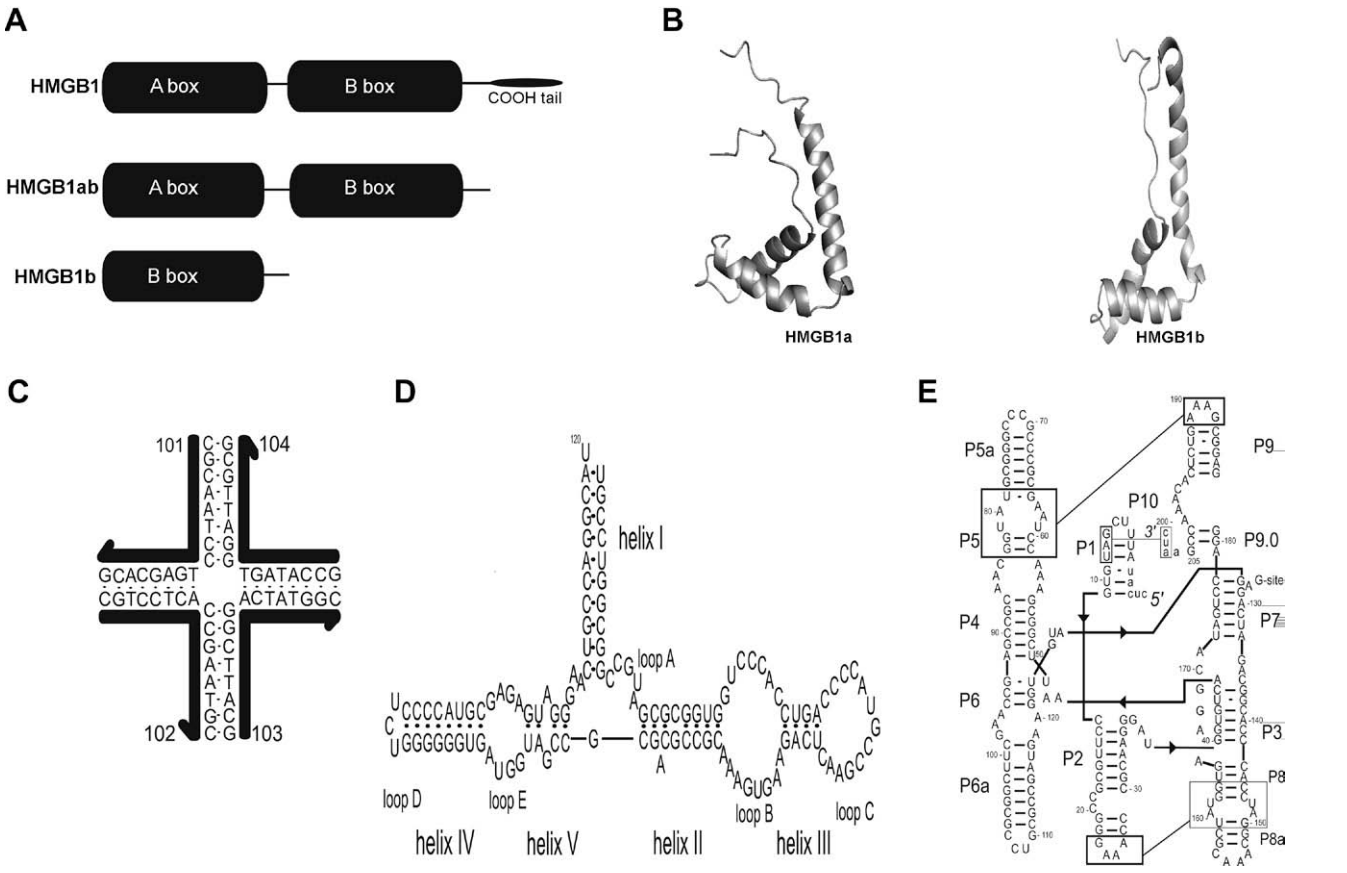

Fig. 1. (A,B) Schematic representation and NMR structure of recombinant HMG box proteins. HMGB1a and HMGB1b structures correspond to PDB coordinate files 1aab and 1hme. (C) Schematic of the four-way junction DNA, J1. (D) Secondary structure of *E. coli* 5S rRNA. (E) Schematic of the group I pre-tRNAIle ribozyme, figure is modified from the model presented by Cech [27].

Materials and methods

HMGB1 protein constructs. HMGB1b and HMGB1b/R26A (R26A), were expressed from the HMGB1b/pHB1 clone that was kindly provided by S.J. Lippard (Department of Chemistry, Massachusetts Institute of Technology). The alanine replacement mutation of R26A was introduced using the Kunkel method [9]. The di-domain protein, HMGB1ab, was cloned and inserted into the pGEX-4T-3 vector as described previously [6]. Recombinant HMG proteins were expressed, purified and characterized by methods described previously [6]. H1 and core histone proteins were kindly provided by Greg Bowman (T.C. Jenkins Department of Biophysics, Johns Hopkins University).

4WJ formation. The 4WJ, J1, was the DNA substrate employed in competitive EMSAs (Fig. 1B) [10]. Oligonucleotide 101 was radiolabeled at its 5′ terminus with [γ-^{32}P]ATP using T4 polynucleotide kinase. The radiolabeled strand (101) was purified with a Bio-Spin 6 column (BioRad). J1 was annealed by mixing the radiolabeled strand (101) with a 5-fold excess of the unlabeled oligonucleotides in 50 mM Tris–HCl (pH 7.5)/10 mM $MgCl_2$. The mixture was lyophilized and suspended in annealing buffer: 50 mM Tris–HCl (pH 7.5) and 10 mM $MgCl_2$, incubated for 2 min at 90 °C and cooled to room temperature.

5S rRNA E. coli. 5S rRNA (120-nt) was purchased from Sigma–Aldrich. 5S rRNA stock samples were diluted in 10 mM Tris–HCl (pH 7.5), 0.1 M NaCl and 1 mM $MgCl_2$ before use.

(L-3) Azoarcus Ribozyme. The (L-3) *Azoarcus* ribozyme was prepared as previously described [11]. The ribozyme was engineered by deleting the first two nucleotides of the intron and the last two guanosines (G205 and G206) at the intron's 3′-end. The nucleotides U4, U5 and U6 were mutated to G4, G5 and C6, respectively. The ribozyme transcripts were gel purified and resuspended in 10 mM Tris–HCl (pH 7.5) and 0.01 mM EDTA before use.

Electrophoretic mobility shift assays (EMSAs). Radiolabeled J1 (12.5 nM) was incubated with HMG proteins in the absence and in the presence of competitor RNA. Each reaction mixture was incubated in binding buffer: 20 mM Tris–HCl (pH 7.5), 100 mM NaCl, 1 mM $MgCl_2$ and 10% (w/v) glycerol in a final volume of 20 μl on ice for 30 min prior to loading onto 15% polyacrylamide gels in 0.5× TAE [20 mM Trisma (pH 8.0), 8 mM acetate, 0.1 mM $MgCl_2$) at 4 °C for ~3.5 h. Gels were dried and analyzed using a Storm PhosphorImager.

Circular dichroism measurements. Circular dichroism (CD) spectra were recorded using an AVIV 202 spectrometer (Aviv Associates, Lakewood, NJ). RNA-HMG binding interactions were determined by recording the change in the CD spectrum of each RNA. Each RNA (1.0 μM) was incubated on ice for 30 min with each HMG protein (10 μM) in binding buffer: 20 mM HEPES, 30 mM NH_4Cl, 0.2 M KCl, 2 mM DTT, 0.5 mM $MgCl_2$ and 10% glycerol at 4 °C. CD spectra were measured in a 0.1 cm path-length quartz cuvette and collected from 305 to 200 nm in 0.5 nm increments at 4 °C.

Ribozyme cleavage assays. Details of ribozyme cleavage assays presented in Supplementary data [11,12].

Results

Competitive Electrophoretic Mobility Assays

Competitive gel shift assays were conducted to detect binding of HMG proteins to the cognate DNA substrate, J1, in the pres-