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An improved method for large-scale preparation of negatively and positively supercoiled plasmid DNA

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Abstract

A rigorous understanding of the biological function of superhelical tension in cellular DNA requires the development of new tools and model systems for study. To this end, an ethidium bromide-free method has been developed to prepare large quantities of either negatively or positively supercoiled plasmid DNA. The method is based upon the known effects of ionic strength on the direction of binding of DNA to an archaeal histone, rHMfB, with low and high salt concentrations leading to positive and negative DNA supercoiling, respectively. In addition to fully optimized conditions for large-scale (> 500 μg) supercoiling reactions, the method has the advantage of avoiding the use of mutagenic ethidium bromide, of applicability to chemically modified plasmid DNA substrates and of producing both positively and negatively supercoiled DNA using a single set of reagents.

Keywords

DNA supercoiling; rHMfB; archaeal histone; positive supercoiling; negative supercoiling; plasmid DNA

DNA supercoiling is ubiquitous in prokaryotic and eukaryotic genomes (1-4) and has clear effects on many cellular functions (5-10). The study of superhelical tension in DNA, however, has been hampered by the paucity of methods to prepare sizeable quantities of supercoiled plasmid DNA as a model system. Several methods exist to prepare either positively (11) or negatively (12,13) supercoiled plasmid DNA, but these methods are limited by low superhelical density, small preparation size, availability of crucial enzymes, or the need to use highly mutagenic ethidium bromide that causes significant nicking of the plasmid DNA. To date, no single method provides both positively and negatively supercoiled plasmid in parallel and biochemically-similar reactions for use in comparative studies of supercoiled DNA.

To address this problem, we modified a positive supercoiling method previously developed in this laboratory (14) to allow the preparation of both positively and negatively supercoiled plasmid DNA. In the original low ionic strength method, topologically relaxed plasmid is bound to tetramers of rHMfB, which constrains positively supercoiled toroids, while compensatory negatively supercoiled plectonemes form in the unbound portion of the DNA

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(Figure 1A). Treatment with topoisomerase then removes the unconstrained plectonemes while leaving the bound toroids intact (Figure 1B), resulting in plasmid with positive supercoiling upon removal of the archaeal protein (Figure 1C). We now expand this method to allow parallel production of negatively supercoiled plasmid. This was achieved by exploiting a published observation (15) that the direction of wrapping of DNA around the archaeal histone rHMfB is dictated by the ionic strength of the buffer. With a switch from low to high ionic strength, the direction of wrapping of DNA around the rHMfB histones is reversed to constrain negative toroids and thus negative supercoiling. We have also added an acid phenol extraction step to remove non-closed-circular (*i.e.*, nicked) plasmid from the preparation.

The method begins with preparation of plasmid substrate, a topoisomerase-containing extract and rHMfB protein. Plasmid pUC18 prepared by alkaline lysis (16) was dialyzed against 3 M NaCl in TE buffer (10 mM Tris, 1 mM EDTA, pH 8) using high molecular weight (100,000 Da) tubing, followed by exhaustive dialysis against TE buffer alone. A topoisomerase-containing extract of chicken erythrocyte nuclei (referred to as chicken blood extract or CBE) was prepared from fresh, sterile, citrate-treated chicken blood (Rockland Immunochemical, Gilbertsville, PA) as previously described (17), aliquoted and stored at −80 °C. One unit (U) of CBE was defined as the volume of extract required to relax 1 μg of pUC18 under standard relaxation conditions (see below). Inactivated CBE for use in preparing sham-treated DNA substrates was prepared by heating CBE to 95 °C for 20 min. Isolation of rHMfB was performed as described previously (18), except that cells were lysed by two passages through an Emulsiflex-C5 homogenizer (Avestin, Ottawa, ON) at 20,000 psi, and a Hi-Prep 16/10 Heparin FF column (GE Healthcare, Piscataway, NJ) was used for affinity purification. Fractions containing rHMfB were dialyzed against 4x rHMfB binding buffer (40 mM Tris, 4 mM EDTA, 8 mM K_2HPO_4, 200 mM NaCl, pH 8.0), and stored at 4 °C.

The first step in the method involves relaxation of negatively supercoiled plasmid (50 μg/mL) by treatment with 1 U CBE/μg plasmid in 200 mM NaCl, 20 mM Tris (pH 8.0), 0.25 mM EDTA, 5% glycerol for 1.5 h at 37 °C. The reaction was adjusted to 1% SDS and 150 μg/mL proteinase K, and incubated at 37 °C for 1.5 h. DNA was further purified by phenol/chloroform/isoamyl alcohol extraction followed by chloroform extraction and ethanol precipitation. After resuspension in TE, the DNA was desalted by passage over a NAP-25 column (GE Healthcare), and quantified spectrophotometrically.

The next step is to determine the optimal proportions of rHMfB and DNA, which is crucial to maximizing the supercoiling; either too little or too much rHMfB will result in lower overall supercoiling (Figure 2A). The proportions of rHMfB and DNA were optimized by mixing 0.5 μg of relaxed plasmid (25 μg/mL final concentration) with varying quantities of rHMfB (0.5-1.5 mass equivalents) in 1xrHMfB reaction buffer in the presence (for negative supercoiling) or absence (for positive supercoiling) of 350 mM potassium glutamate. Unrestrained compensatory supercoiling was removed by adding 10 U of CBE per μg of plasmid following adjustment of the buffer by addition of 1/10 volume of 590 mM Tris, 8 mM K_2HPO_4, 22 mM EDTA, 100 mM NaCl, pH 8.0 and further addition of 1/10 volume of either 1.4 M K-glutamate (for negative supercoiling) or water (for positive supercoiling). Reactions were incubated again for 1.5 hr at 37 °C. DNA was purified as described earlier and the degree of supercoiling assessed by one-dimensional agarose gel electrophoresis. Batches of ≥500 μg supercoiled plasmid DNA were then prepared by scaling the optimized reactions for the larger quantities and volumes.

Several salt species were tested for efficacy in this protocol, with K-glutamate found to be the most consistent for production of large quantities of negatively supercoiled plasmid

(Table 1). Of the salt species that did not result in supercoiled plasmid, all were found to interfere with activity of the topoisomerase added to remove compensatory supercoils (data not shown).

The proportion of nicked plasmid was minimized to <5% using a modification of the acidic phenol extraction method of Zasloff *et al*. (19). Plasmid was resuspended in TE and desalted on a NAP-25 column. The DNA solution (<0.5 mg/mL) was adjusted to 75 mM NaCl and 50 mM sodium acetate, pH 4, followed by addition of 1 volume of phenol, previously equilibrated with sodium acetate (50 mM, pH 4), and vortexing for 5 min. Following centrifugation (6,000 × g, 5 min), the aqueous layer was immediately removed and neutralized by addition to 1/10 volume of 2 M Tris, pH 8. Residual phenol was removed by chloroform extraction, and plasmid was precipitated with 1 volume isopropanol and resuspended in TE. Abasic sites were quantified by gel electrophoresis following treatment of plasmid DNA with 0.1 M putrescine (pH 7, 37 °C, 30 min) to convert abasic sites to strand breaks (20). By limiting exposure of the plasmid to acidic conditions, we are able to reduce the fraction of nicked plasmid in the preparation (Figure S1A) without DNA depurination (Figure S1B).

After purification, topoisomer content and direction of supercoiling in the substrates were determined by one- and two-dimensional gel electrophoresis as described in detail elsewhere (21), with the fraction of nicked molecules determined by gel electrophoresis in the presence of ethidium bromide (21). As shown in Figure 2, controlled variation of the salt concentration leads to a similar degree of plasmid supercoiling of both signs, with >50% of the molecules possessing linking number changes (ΔLk) from ±7 to 15 (Fig. 2 and data not shown). This yields superhelical density (σ) values of ± 0.03-0.06 in plasmid pUC18 (2686 bp). The maximally resolved ΔLk for negatively supercoiled plasmid in Figure 2C is −10, while that for positively supercoiled plasmid runs up to +15 (data not shown). This degree is supercoiling is similar to that reported using the published methods for preparing positively supercoiled (14) and negatively supercoiled plasmid DNA (13). Individual topoisomer populations can be isolated by extraction of plasmid DNA from excised agarose gel bands or by gradient centrifugation.

Here we describe an improved method for the preparation of large quantities of plasmid DNA containing high levels of either positive or negative supercoiling. In addition to fully optimized conditions for large-scale (> 500 μg) reactions, the method has the advantages of avoiding the use of mutagenic ethidium bromide, of applicability to chemically modified plasmid DNA substrates, and of producing both positively and negatively supercoiled DNA with high superhelical density and low levels of nicking using a single set of reagents. The resulting DNA substrates are ideal for sensitive biochemical and biophysical studies of DNA supercoiling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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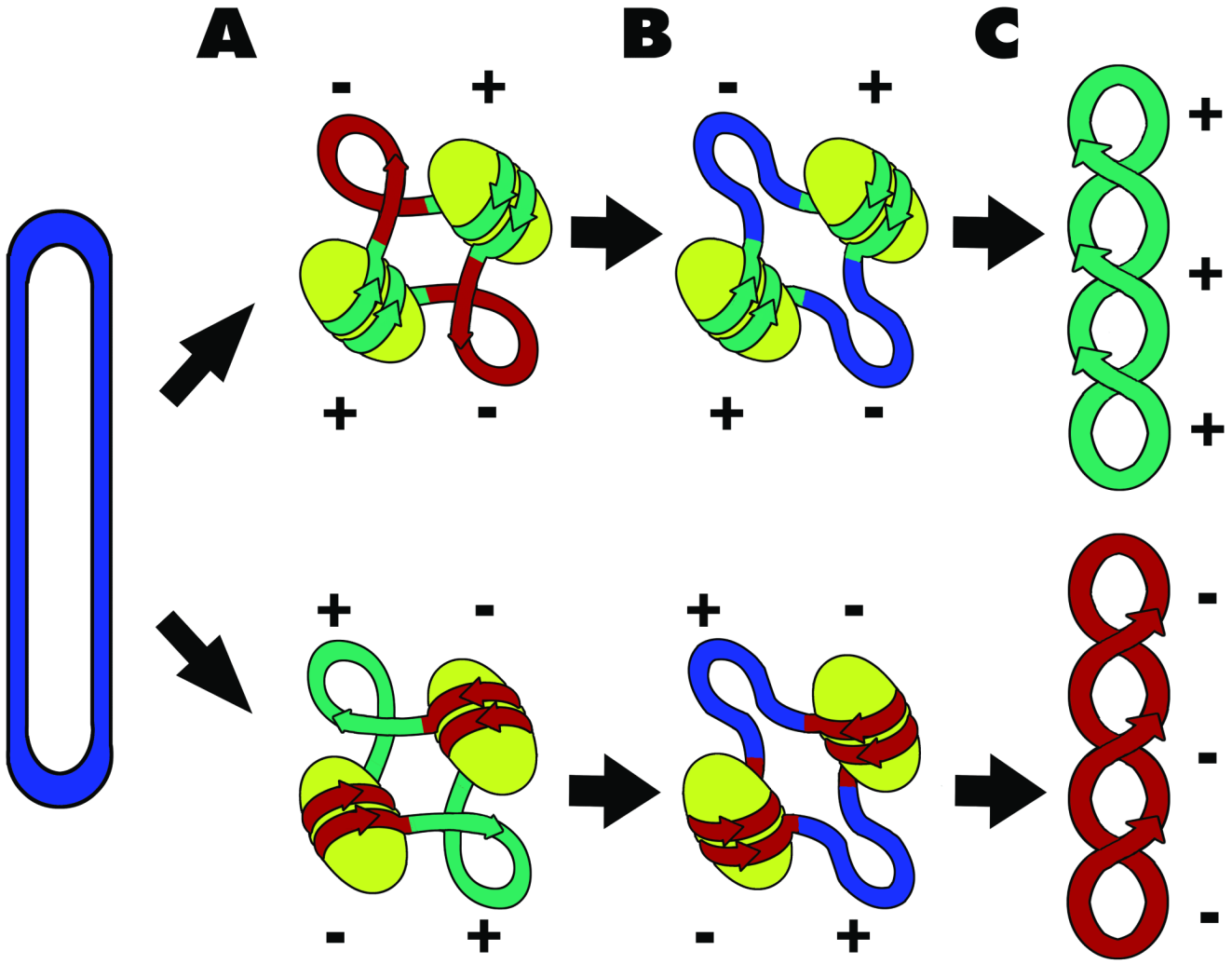

Figure 1. Preparation of supercoiled plasmid

(**A**) Relaxed, closed circular plasmid binds to histone rHMfB (blue) in the absence (top) or presence (bottom) of high concentrations of salt to induce either positive (top) or negative (bottom) constrained supercoils. Compensatory plectonemic supercoils of opposite sign form in the protein-free regions of DNA. (**B**) Topoisomerase activity removes compensatory supercoiling, while rHMfB-constrained supercoiling remains intact. (**C**) Removal of rHMfB protein releases constrained supercoiling of either sign as plectonemic supercoiling in the product plasmid.

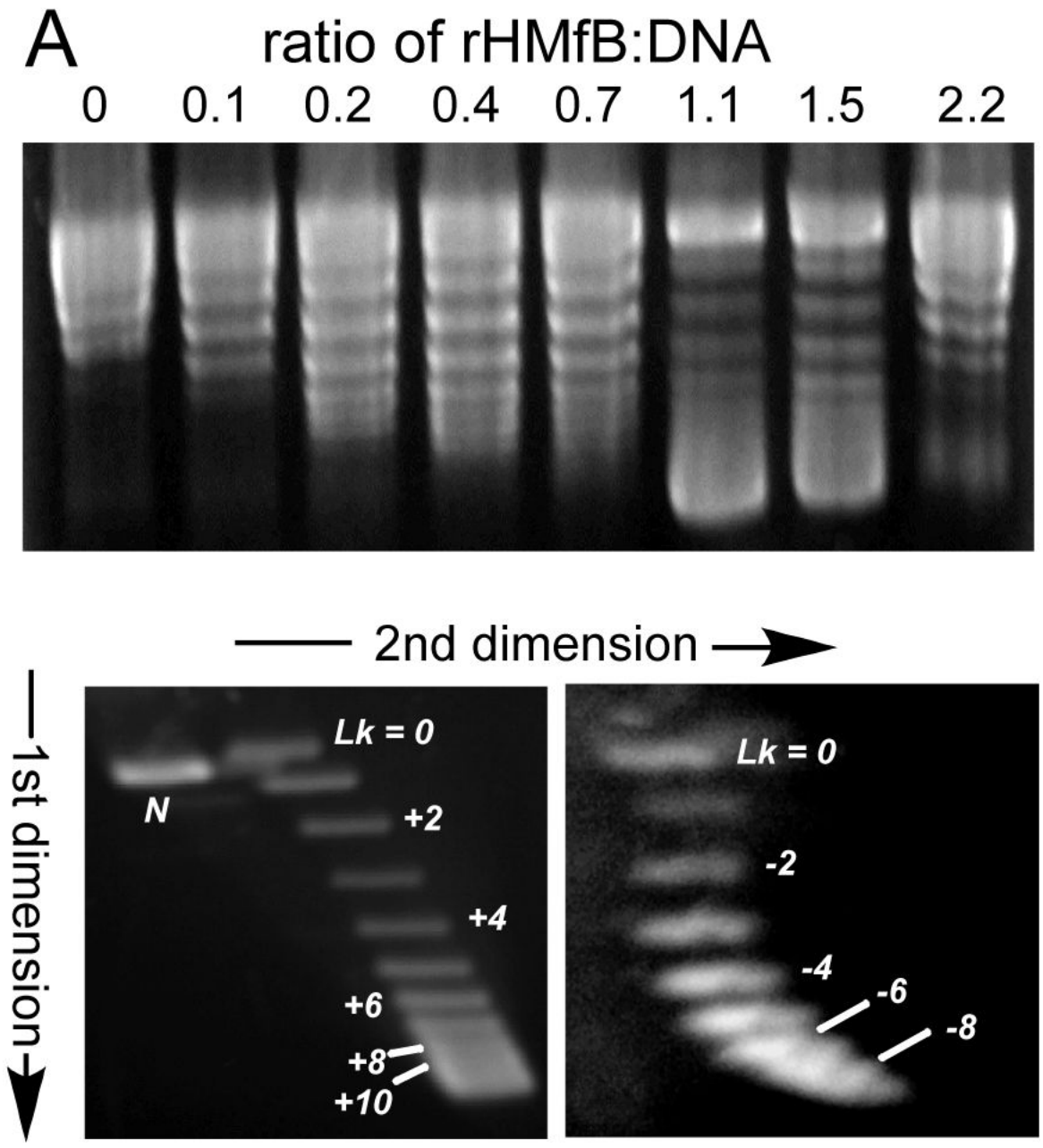

Figure 2. Electrophoretic analysis of supercoiled plasmid DNA

(**A**) One-dimensional gel analysis of plasmid topoisomers resulting from supercoiling optimization performed using varying proportions of rHMfB protein and DNA (ratio of mass units). Optimal supercoiling was found to occur with a protein-to-DNA ratio of ~0.8 in this study. (**B**) Two-dimensional gel analyses of the highly supercoiled plasmid molecules resulting from large-scale reactions in either low salt (**left**; positive supercoiling) or 350 mM potassium glutamate (**right**; negative supercoiling). N, nicked plasmid molecules; linking number changes are noted in each image. One- and two-dimensional agarose gel electrophoresis was performed as described elsewhere (14,21).

Table 1

Effect of salt on topoisomerase activity and supercoiling

Salt added[a]	Relaxation[b]	Supercoiling[c]
Control (no salt)	Y	POS
KCl	N	0
NaCl	N	0
K-Acetate	N	0
Na-Acetate	N	0
KPO_4	N	0
K-Glutamate	Y	NEG
Na-Glutamate	Y	NEG
K-Aspartate	Y	NEG

[a] Salt species added to 350 mM final concentration during relaxation of compensatory supercoils.

[b] Relaxation of negatively supercoiled plasmid in the absence of rHMfB to assess effects of salt on topoisomerase activity. N, no relaxation; Y, relaxation similar to low salt conditions.

[c] Direction of supercoiling following the complete protocol. 0, no supercoiling induced; POS and NEG, positive and negative supercoiling, respectively.