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Chapter 2

Analysis of DNA Topoisomers, Knots, and Catenanes by Agarose Gel Electrophoresis

Stephen D. Levene

Abstract

Agarose gel electrophoresis is by far the most widely used method for characterizing the topological state of DNA molecules. Although this technique has been used for more than 30 years, the physical mechanism underlying the resolution of topological states remains poorly understood. However, electrophoretic methods remain the most robust and precise techniques for determining the local unwinding of DNA induced by the binding of proteins and small-molecule ligands, analyzing conformational transitions in duplex DNA, measuring changes in helical repeat that accompany shifts in environmental conditions, and characterizing knotting and linking in duplex DNA.

Key words: Topoisomers, electrophoresis, catenanes, linking number, DNA knots.

1. Introduction

Linking-number topoisomers, knots, and catenanes (linked circles) are topological forms of circular DNA that have distinct three-dimensional conformational properties.¹ The analysis of DNA topology has played a major role in molecular biology, yielding essential insights into the mechanisms of topoisomerases and related proteins such as site-specific recombinases. The mechanistic feature common to all of these systems is that they

¹ We follow typical usage here, in which the term “topoisomer” refers specifically to unknotted or unlinked DNA circles with distinct linking-number values. However, molecules that differ in knot and catenane type are also properly regarded as topoisomers because these forms are identical apart from differences in their respective topological states.

pass DNA strands through one another; however, there is extraordinary variation in the details of strand exchange. The resulting distributions of product topologies provide strong mechanistic clues that illuminate the biochemical details of DNA reorganization during the reaction pathway (1–3). For example, **Fig. 2.1** shows a mechanistic model for members of the tyrosine-recombinase superfamily, which includes λ int, Cre, and Flp. The mechanism consists of protein binding to a circular DNA molecule, cutting of the DNA at two specific sites, and subsequent exchange and rejoining of the cleaved ends, producing a specific knot (**Fig. 2.1A**) or catenane (**Fig. 2.1B**). In systems that act on recombination-target sites having sequence asymmetry, only one juxtaposition of sites leads to productive recombination. Thus, in the example shown in **Fig. 2.1A**, recombination of an unknotted supercoiled plasmid bearing inversely oriented sites leads to a knotted product, whereas recombination of directly oriented sites on a similar superhelical DNA in **Fig. 2.1B** generates a catenane.

On a circular DNA molecule, the pair of target sites bound by the topoisomerase or recombinase during site synapsis divides the DNA contour into two distinct domains. For a particular conformation of the synaptic complex, the number of DNA crossings in the product is a function of the number of interdomainal windings. In the case of an intramolecular tyrosine-recombinase reaction taking place on an unknotted circular DNA, the number of irreducible crossings in the products is proportional to the number of superhelical turns that are trapped between the sites (**Fig. 2.1**). The knot or catenane types that are generated in a particular enzymatic reaction are a signature for a specific enzymatic mechanism. This is generally posed as an inverse problem, in which one deduces the enzymatic mechanism from a particular distribution of superhelical, knotted, or catenated products (2, 3). Solution of the inverse problem is not as difficult as it may appear; although the space of possible knots grows exponentially with the number of irreducible crossings (there are 1,388,705 prime knots with 16 or fewer crossings), only a limited subset of knots is generated by an enzyme system (**Fig. 2.2**).

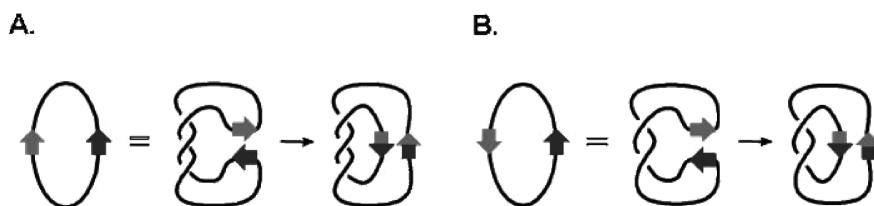


Fig. 2.1. Tyrosine recombinases can knot and link circular DNA molecules. (A) Knotting of DNA by recombination of inversely repeated sites. **(B)** Linking of product DNA circles by recombination of directly repeated sites.

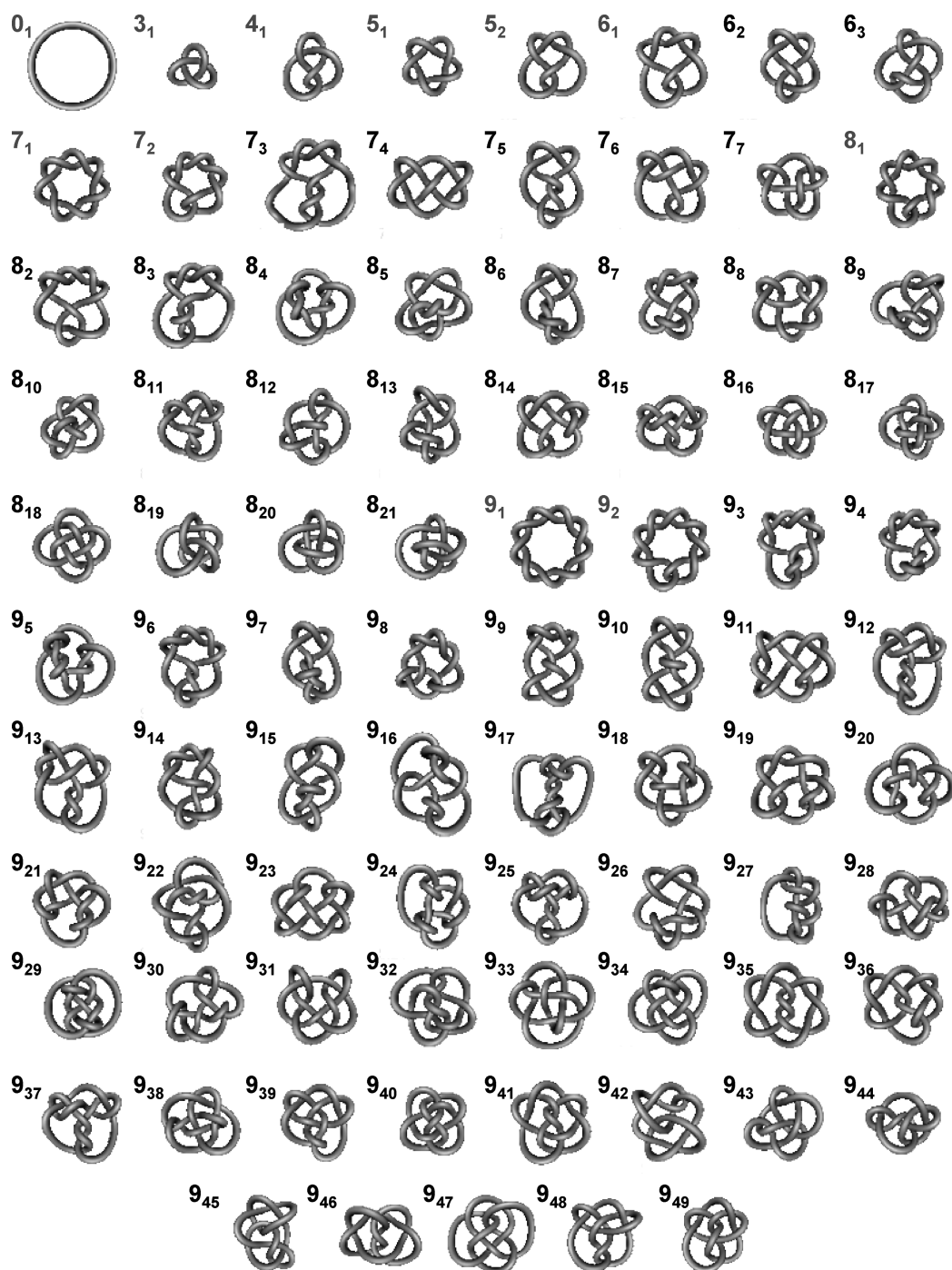


Fig. 2.2. **Prime knots.** Minimal diagrams of all prime knots containing up to nine irreducible crossings generated with the program *KnotPlot* (4). Each knot is labeled according to the notation of Alexander-Briggs. Knots that are commonly observed as topoisomerase and recombinase products are indicated by *gray* labels.

1.1. Gel Electrophoretic Analysis of Linking-Number Topoisomers

Supercoiling is parameterized in terms of the linking number of duplex strands, Lk , which is a topologically invariant quantity for closed-circular DNA molecules (5). The Lk value trapped by cyclization, ligation at a nick, or topoisomerase activity is an integer equal to the sum of two geometric variables, twist, Tm , and writhe, Wr , which fluctuate at thermal equilibrium. For plasmid-sized DNAs, covalent closure in vitro generates a Gaussian distribution of species typically composed of several Lk topoisomers. Topoisomers can be analyzed by gel electrophoresis using one-dimensional (1-d) and two-dimensional (2-d) agarose gels (6–9). Although the physical basis for the electrophoretic separation of topoisomers is poorly understood, it has been proposed that gel mobility is largely a function of the average DNA writhe, $\langle Wr \rangle$ (6–14). The actual value of Lk is rarely of interest; instead, changes in this parameter are measured by comparing Lk distributions obtained under different conditions, for example, in the presence and absence of a perturbing ligand. Both 1-d and 2-d methods offer particular advantages in different experimental contexts.

1.1.1. One-Dimensional Agarose Gel Analysis of Topoisomers

The 1-d gels offer a straightforward approach for quantitating differences in DNA helical repeat and are less technically challenging than the 2-d methods. The experiment outlined in **Fig. 2.3** shows an example of 1-d gel analysis of the helical-repeat change induced in duplex DNA by formation of a RecA strand-exchange complex (15, 16). Under appropriate conditions, *Escherichia coli* RecA protein forms a stable complex with the duplex and a single-stranded DNA complementary to one of the duplex strands; in this complex, the duplex is strongly unwound. Unwinding is detected by a shift in the center of the plasmid/strand-exchange complex Lk distribution, $\langle Lk \rangle$, relative to that in the absence of the complex, after relaxation with topoisomerase I.

1.1.2. Two-Dimensional Agarose Gel Analysis of Topoisomers

The 2-d gels are a method of choice for characterizing supercoiling-dependent localized changes in helical structure such as cruciform extrusion, formation of Z-DNA, and local denaturation (7). Unlike 1-d gels, which are capable of resolving topoisomers only over a narrow ΔLk interval (typically a maximum of ten species), 2-d gels can resolve more than 20 individual topoisomers (**Fig. 2.4**). Moreover, under appropriate conditions, topoisomers of similar $|\Delta Lk|$, but opposing sign are well resolved on 2-d gels; these species comigrate in 1-d electrophoresis. The 2-d methods exploit the strong dependence of topoisomer mobility on electrophoresis conditions usually by running the gel without an intercalating agent such as chloroquine or ethidium in the first dimension, but including an intercalator in the gel and running buffer in the second dimension.

The example shown in **Fig. 2.4** illustrates the analysis of supercoiling-dependent DNA structure using 2-d agarose gel electrophoresis. Here, the discontinuity in the arc-like pattern of

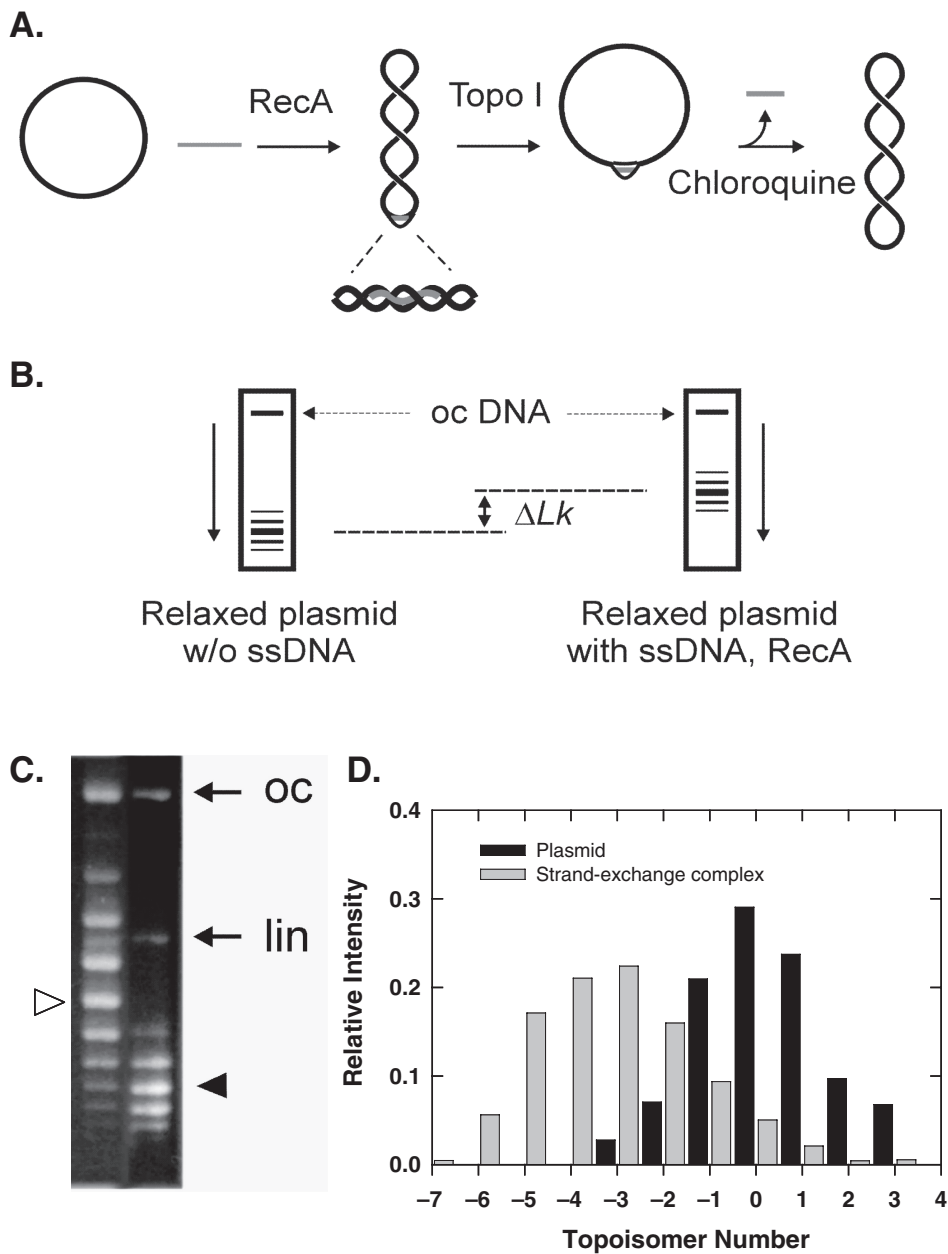


Fig. 2.3. **One-dimensional analysis of topoisomers.** (A) Incubation of relaxed plasmid DNA with an ssDNA-containing RecA filament results in the formation of a paranemic ssDNA–dsDNA complex bound to RecA. Relaxation with topoisomerase I removes extraneous supercoils from the locally underwound paranemic complex. The change in DNA twist accompanying formation of the complex is revealed by agarose gel electrophoresis in the presence of the intercalator chloroquine, which also disrupts the complex through positive supercoiling. (B) Measurement of the linking difference, ΔLk , by agarose gel electrophoresis. The distribution of topoisomers is quantitated from digital images of gel lanes containing native plasmid DNA and plasmid relaxed under conditions in which the complex is formed. The values of Lk used in computing ΔLk are those corresponding to the most probable topoisomer in each distribution. (C) The 1-d agarose gel analysis of topoisomer distributions for the native plasmid (*right lane*) and strand-exchange complex (*left lane*). Positions of the most probable topoisomers are given by the filled and open arrowheads. (D) Equilibrium distributions of topoisomers in the relaxed plasmid and the strand-exchange complex. (Adapted with permission from Ref. 16. Copyright 2005 American Chemical Society.)

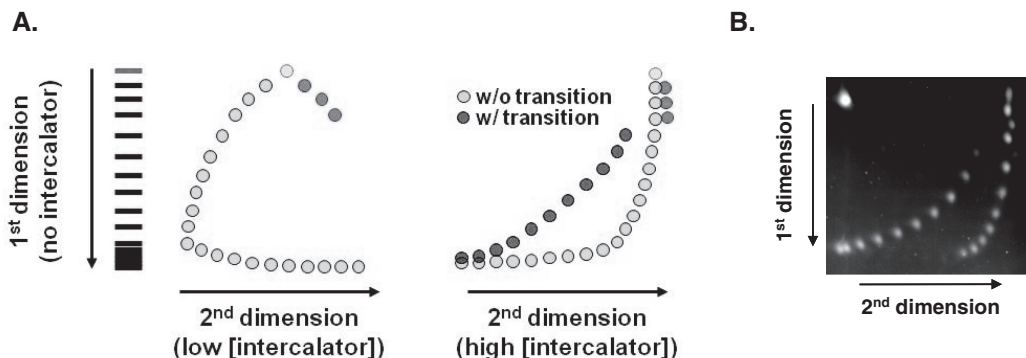


Fig. 2.4. **Two-dimensional analysis of topoisomers.** (A) Principle of 2-d gel electrophoresis. Topoisomer separation achieved by a typical 1-d gel in the absence of an intercalating agent is shown on the far left. After equilibrating the gel with running buffer containing an intercalator, subsequent electrophoresis in the orthogonal direction allows resolution of bands that comigrate in the first dimension. If sufficient intercalator is present to drive all of the topoisomers into a (+) supercoiled state (high [intercalator]), a concave arc-like pattern of topoisomer bands is observed. The expected behavior for a plasmid undergoing a structural transition is indicated by the *dark gray* bands in the high [intercalator] example, which follows a discontinuous arc pattern. (B) The 2-d agarose gel analysis of pUC8S1F2, which contains a 60-bp inverted-repeat sequence shown to undergo a cruciform transition (5). Electrophoresis was carried out without intercalator in the first dimension and in 3.0 $\mu\text{g/ml}$ chloroquine in the second dimension. Note the discontinuity in the 2-d gel pattern at $\Delta Lk = -10$. (Reproduced from Ref. 5.)

topoisomers seen in **Fig. 2.4B** is characteristic of the formation of an extrahelical DNA structure at a critical level of (–) supercoiling, in this case, a cruciform structure (5). Extrusion of the cruciform sharply increases ΔLk , which abruptly shifts the pattern of topoisomers toward the origin of the first dimension. This behavior is not normally detected in 1-d gels because of the overlap between shifted and unshifted topoisomer bands.

1.2. Gel Electrophoretic Analysis of DNA Knots and Catenanes

Because of the great sensitivity of gel mobility to supercoiling, knotted and catenated circular DNA must be nicked prior to electrophoresis to obtain readily interpretable gel patterns. Otherwise, separations of knotted and catenated circular DNAs take place in much the same way as 1-d gel electrophoresis of Lk topoisomers (*see Section 1.1.1*), apart from the use of intercalating agents, which do not affect the relative mobilities of nicked knots and catenanes. At sufficiently low field strengths, spacing between successive knot and catenane bands is nearly constant with the mobility of a particular species effectively proportional to the number of irreducible crossings (**Fig. 2.5**). There is very little compression of knot or catenane ladders at high crossing number, although small differences in mobility can be detected for distinct knot types having the same number of irreducible crossings at low crossing number (17). Separations of distinct knot types with identical crossing number can be achieved using 2-d gel- electrophoresis methods (18).

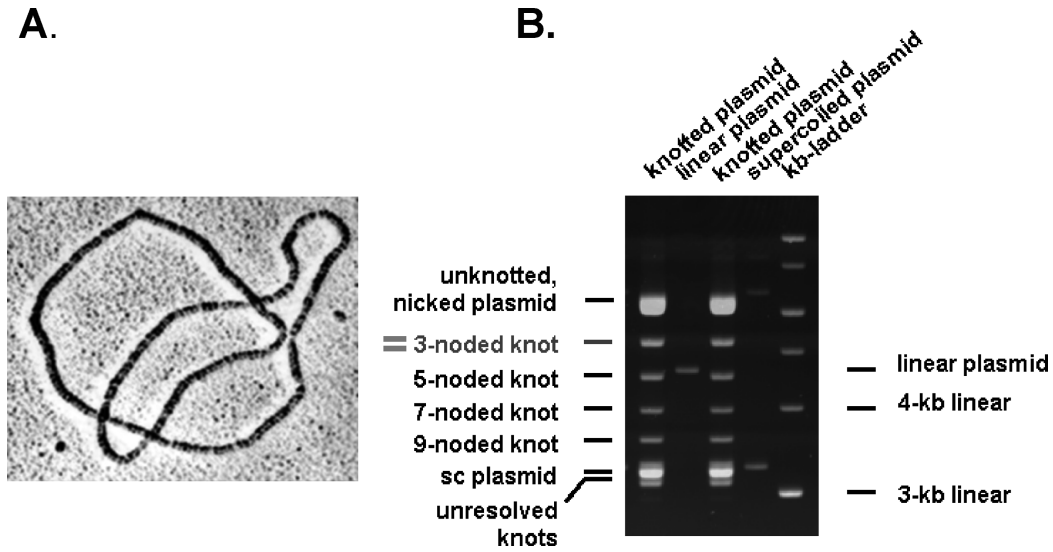


Fig. 2.5. **Physical characterization of knotted DNAs.** (A) Electron micrograph of a three-noded DNA knot visualized after coating with *E. coli* RecA protein and rotary shadowing. (B) Separation of DNA torus knots (and catenanes) by agarose gel electrophoresis. Gel electrophoresis can separate knots (and catenanes) by their minimal crossing numbers, but is otherwise relatively insensitive to knot type. Samples such as in (A) are obtained by isolating DNA molecules from specific bands in the gel (Micrograph reproduced with permission from Krasnow et al. (1983) *Nature* 304, 559–560. Copyright 1983 Nature Publishing Group).

Despite the high resolution of gel techniques, the quantitative relationship between knot/catenane mobility and DNA topology remains poorly understood. Thus, additional experiments are generally needed to definitively establish the DNA topology that corresponds to a particular gel band (e.g., the four-noded knot and four-noded torus catenane have similar mobilities). The most reliable (though by no means foolproof) technique for identifying knot topology is electron microscopy of RecA-coated DNA (*see* Fig. 2.5A) (19). Although potentially error-prone, it is more typical for bands to be assigned by running samples in parallel with a ladder of known knots or catenanes generated by recombination or topoisomerase activity (2).

2. Materials

2.1. Plasmid DNA

Most of the plasmids used in our laboratory are derived from the pGEM series of cloning vehicles available from Promega, Inc. These pGEM derivatives have a very high copy number, yielding in the range of 4–10 mg of plasmid per liter of culture. The inventory of constructs we have developed for use as substrates

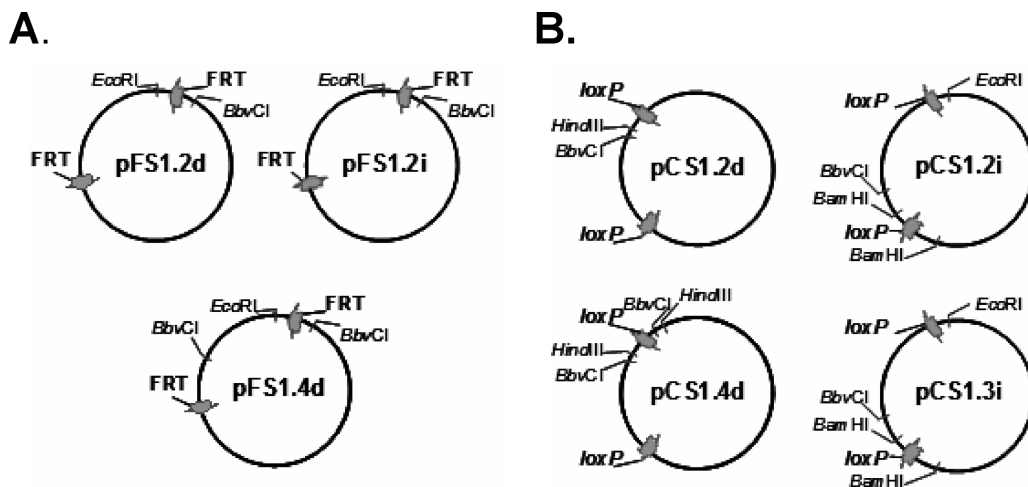


Fig. 2.6. **Plasmid DNAs.** Examples of plasmid DNAs used as Flp-recombination (**A**) and Cre-recombination (**B**) substrates. Plasmids pFS1.2d and pFS1.2i are identical 3759-bp DNAs containing directly and inversely oriented minimal FRT sites, respectively. Plasmid pFS1.4d (3763 bp) is identical to pFS1.2d except for the presence of a second *Bbv*CI recognition site in the small plasmid domain between the FRTs. Plasmid pCS1.4d (3657 bp) is identical to pFS1.2d (3625 bp), except for the presence of a second *Bbv*CI recognition site in the large plasmid domain between the loxP sites. Locations of *Bbv*CI and unique *Eco*RI or *Hind*III sites in both plasmids are as indicated. (Reproduced from Ref. 22.)

in site-specific recombination is too large to describe here, however, maps of several examples are shown in **Fig. 2.6**. Aside from the presence of loxP or FRT sites, the most notable feature is the strategic placement of *Bbv*CI restriction sites, which function as site-specific nicking sites when the plasmid is incubated with either of the mutant enzymes Nt.*Bbv*CI or Nb.*Bbv*CI (20). *Bbv*CI is a heterodimer; in these mutant enzymes, one of the two subunits is present in a catalytically inactive form. Thus, the mutant endonucleases recognize the cleavage site normally, but act on only one of the strands to generate a site-specific nick. The yield of nicked DNA in a digest containing excess nicking enzyme is generally 90–95%, with near-negligible contamination from linear DNA. Therefore, this method is much preferred to more traditional methods relying on limited nicking by DNaseI in the presence of ethidium bromide.

Note that DNA substrates containing direct repeats of recombination-target sites generate unlinked circles or catenanes as recombination products (**Fig. 2.1**). Depending on the information that is needed, site-specific nicking sites can be introduced into the plasmid domains corresponding to either of the product circles. If only one nicking site is present on a direct-repeat substrate, then the product after nicking will be a hemi-nicked catenane, which will complicate the electrophoretic analysis relative to that for a fully nicked catenane.

2.2. Recombination Proteins and Topoisomerases

Proteins can be obtained from a number of different sources, and we have been successful in isolating and purifying many of these proteins in our laboratory according to published procedures (21, 22); however, we have also relied on commercial preparations in some cases. Wheat-germ topoisomerase I, human topoisomerase II, Cre, Nb.*Bbv* CI, and Nt.*Bbv* CI are all available commercially from various suppliers (*see* **Note 1**).

2.3. Solutions (see Note 2)

1. Int-recombination buffer (+Mg²⁺): 20 mM Tris-HCl, 50 mM NaCl, 20 mM KCl, 10 mM MgCl₂, pH 7.6.
2. Int-recombination buffer (-Mg²⁺): 10 mM Tris-HCl, 50 mM NaCl, 5 mM spermidine, 1 mM Na₂EDTA, pH 7.5.
3. FLP reaction buffer: 25 mM HEPES, 100 mM NaCl, 1 mM Na₂EDTA, 25% (w/v) ethylene glycol, pH 7.5.
4. Cre reaction buffer: 10 mM Tris-HCl, 50 mM NaCl, 10 mM MgCl₂, 1 mM dithiothreitol, pH 7.9.
5. Topoisomerase I buffer: 50 mM Tris-HCl, 50 mM NaCl, 1 mM Na₂EDTA, 1 mM DTT, 0.1 mg/ml bovine serum albumin, pH 7.5.
6. TE buffer: 10 mM Tris-HCl, 1 mM Na₂EDTA, pH 8.0.
7. TBE electrophoresis buffer: 50 mM Tris-borate, 1 mM Na₂EDTA, pH 8.5. The conductivity of each batch of electrophoresis buffer is monitored to ensure reproducibility; typical values are in the range 8.0–9.0 mS/cm.
8. Agarose: 0.7–1.0% agarose gels are prepared from Sea-Kem[®] LE agarose in TBE buffer. Small quantities of low-melting agarose, such as SeaPlaque GTG, are used as an adhesive in 2-d gels. Both can be obtained from the Lonza Group, Ltd.
9. Chloroquine phosphate stock solution: 10 mg/ml.
10. Ethidium bromide stock solution: 5 mg/ml.
11. 3 M sodium acetate, pH 5.2.
12. TE-saturated phenol.
13. TE-saturated butanol.
14. Chloroform-isoamyl alcohol (24:1).

3. Methods

3.1. Plasmid Purification

1. Isolate plasmid DNA from *E. coli* strain DH5 α by using the alkaline lysis method followed by polyethylene-glycol precipitation (23).

2. Resuspend the DNA pellets in TE buffer and distribute into aliquots containing 100–250 μg DNA.
3. Add 3 volumes of absolute ethanol. (These DNA slurries can be stored in screw-capped microcentrifuge tubes at -20°C until use.)
4. Recover the plasmid DNA from the slurry by pelleting at $15,000g$ for 20 min.
5. Resuspend the pelleted DNA at a concentration of 1 mg/ml in TE buffer.
6. Determine the relative proportion of nicked to superhelical plasmid DNA by electrophoresis in a 0.8% agarose minigel. (If the proportion of nicked DNA is greater than 20%, then the plasmid should be repurified by banding on a 3-ml ethidium bromide CsCl gradient. After extraction of the ethidium bromide with TE/CsCl-saturated butanol, the CsCl should be removed by extensive microdialysis against TE buffer.)

3.2. Preparation of Lk-Topoisomer Ladders

1. Resuspend 1–10 μg of plasmid DNA, along with ethidium bromide or chloroquine phosphate from appropriate stock solutions, if required, in topoisomerase I buffer.
2. Add wheat-germ or yeast topoisomerase I at 5–10 U/ μg of DNA (*see* **Note 3**).
3. Incubate for 30–60 min at constant temperature (*see* **Note 4**).
4. Add 0.1 volume of 10% SDS.
5. Add 0.1 volume of 3 M sodium acetate, pH 5.2, and extract twice with phenol, once with phenol:chloroform (50:50), and once with chloroform. Extract at least twice with TE-saturated butanol to remove traces of the intercalator.
6. Precipitate the DNA with 3 volumes of absolute ethanol.
7. Analyze the topoisomers using 1-d agarose gel electrophoresis (*see* **Section 3.5** and **Note 5**).

Equilibrium distributions of topoisomers are Gaussian, about an average value of ΔLk determined by the concentration of chloroquine. To generate a quasiuniform distribution of ΔLk values for 2-d gel electrophoresis, individual topoisomer distributions are pooled after 1-d analysis (step 7).

3.3. Preparation of Torus-Knot and Torus-Catenane Ladders by Phage- λ Integrative Recombination

1. Incubate 2 μg of (–) supercoiled DNA, 200 ng of Int, and 240 ng of IHF in 20 μl of Int-reaction buffer at 22°C for 30 min.
2. Quench the reaction by incubating it at 65°C for 5 min.
3. If using $+\text{Mg}^{2+}$ conditions, add 5 U of Nb.*Bbv*CI or Nt.*Bbv*CI to the reaction mixture and incubate at 37°C for

1 h. If using $-Mg^{2+}$ conditions, supplement the reaction to 10 mM $MgCl_2$, add 10 U of Nt.*Bbv*CI or Nt.*Bbv*CI and incubate at 37°C for 1 h.

4. Extract twice with phenol, once with chloroform–isoamyl alcohol, and once with chloroform.
5. Recover the DNA by precipitation with 3 volumes of absolute ethanol and resuspend in 20 μ l of TE buffer.

**3.4. Preparation of
Twist-Knot Ladders
Using Type-II
Topoisomerases (see
Note 6)**

1. Preincubate 2 μ g of plasmid DNA in 20 μ l of Int-recombination ($+Mg^{2+}$) buffer at 37°C for 10 min.
2. Add 4.5 μ g of T2 topoisomerase and incubate for an additional 15 min at the same temperature.
3. Quench the reaction by heating to 65°C for 10 min.
4. Add 5 U of Nb.*Bbv*CI or Nt.*Bbv*CI to the reaction mixture and incubate at 37°C for 1 h.
5. Extract twice with phenol, once with chloroform–isoamyl alcohol, and once with chloroform.
6. Recover the DNA by precipitation with 3 volumes of absolute ethanol and resuspend in 20 μ l of TE buffer.

**3.5. One-Dimensional
Gel Electrophoresis
(see Note 7)**

1. Prepare a 0.8% agarose–TBE gel by suspending 0.8 g of low-EEO agarose per 100 ml in TBE buffer. Weigh the agarose plus buffer (including the flask) prior to heating the suspension in a microwave.
2. Heat the suspension to just short of the boiling point (make certain that the suspension does not boil over). Repeat until the agarose dissolves.
3. Place the agarose solution in a 65°C water bath for at least 10 min.
4. Prepare a casting tray and comb. For optimum resolution, use the thinnest possible comb and ensure that the comb clears the tray by at least 1–1.5 mm.
5. Reweigh the flask containing the agarose solution and add back any water driven off during heating. Add ethidium or chloroquine from stock solution, if required, to the agarose prior to casting the gel. Avoid bubbles when pouring the agarose and cover the tray with plastic wrap to minimize dust contamination. Allow the gel to set at room temperature for at least 1 h.
6. Transfer the solidified gel to an electrophoresis tank and fill with TBE buffer to just cover the gel (approximately 1 l). If the gel contains an intercalator, add an identical concentration of the intercalator to the buffer. Load 5–10 μ l samples plus a 1 kbp ladder lane.

7. Run the gel at 2–2.5 V/cm for 30–60 min *without* buffer circulation to electrophorese the DNA into the gel. If an intercalating agent is used, stop the electrophoresis and lay a thin glass plate over the gel to secure the gel to the casting tray. Initiate buffer circulation by turning on the magnetic stirrer and resume electrophoresis.
8. Run the gel for 20–24 h, circulating the buffer and shielding the apparatus from light if an intercalator is present.
9. If the gel does not contain an intercalator, continue to step 10. Otherwise, remove the intercalator by soaking the gel twice in 0.5 M NaCl for 1 h with agitation. Pour off the NaCl and soak the gel twice in ddH₂O.
10. Stain the gel with ethidium bromide (0.5 µg/ml in ddH₂O) for 20 min or with SYBR Green/Gold per manufacturer's instructions. Destain the gel with ddH₂O for 20–30 min.

3.6. Two-Dimensional Gel Electrophoresis of Lk Topoisomers

1. Prepare a 20-cm-long 0.8% agarose–TBE gel without an intercalating agent according to steps 1–6 described in **Section 3.4**.
2. Apply a minimum of 1.5 µg of pooled topoisomers (*see Section 3.2*) to each of two lanes on a 1-d gel. Include at least one empty gel lane between the samples.
3. Run the 1-d gel under the conditions described in **Section 3.4**.
4. Prepare a 20 × 20 cm slab gel without wells.
5. Divide the first-dimension gel along one edge of a lane containing the topoisomers. Stain the gel with ethidium or SYBR dyes as described above and visualize the topoisomer bands with a transilluminator. If the bands are well resolved in the first dimension, excise the parallel to topoisomer lane from the unstained portion of the gel and trim to the minimum dimensions of the gel pattern, if desired.
6. Prepare 10–20 ml of low-melting agarose and cool to 50°C in a water bath. Rotating the excised gel lane 90 degrees along its long axis, fix the first-dimension topoisomer lane to one edge of the 20 × 20 cm gel using the low-melting agarose. Cool for at least 30 min, and then transfer the composite gel to an electrophoresis tank.
7. Run the gel at 2–2.5 V/cm with buffer recirculation for 24–48 h in TBE buffer containing chloroquine. Protect the apparatus from light. If running for more than 24 h, replace the buffer at least once during this period.
8. Stain and destain the gel according to steps 9 and 10 in **Section 3.5**.

3.7. Gel Analysis and Quantitation

Quantitation of the DNA should be performed using a cooled CCD camera. We perform routine quantitation using an Alta U32 cooled-CCD camera (2148×1472 6.8 μm pixels, Apogee Instruments). The camera operates in 16-bit mode (1:65,536 dynamic range), although the practical dynamic range is between 13 and 14 bits (1:8192–1:16,384). We quantitate individual bands by analyzing the digital image using ImageQuant software (GE Healthcare). For ethidium-stained gels, we use a FirstLight UV transilluminator (UVP), which has highly uniform illumination across the full filter area. For gels stained with SYBR dyes, we visualize the DNA using the Dark Reader visible light transilluminator (Clare Chemical). High-quality interference bandpass filters centered at each dye's emission wavelength (Andover Corp.) are mounted to the CCD camera's objective lens (Schneider Optics).

4. Notes

1. Wheat-germ topoisomerase I is available from Promega, human topoisomerase II from USB Corporation, and Cre, Nb.*Bbv*CI, and Nt.*Bbv*CI from New England Biolabs.
2. All aqueous reagents should be 0.22- μm filtered. Tris buffers should be standardized according to their pH values at 22°C.
3. Topoisomerase I should be assayed periodically to confirm unit activity. The standard unit definition is the amount of enzyme required to relax 1 μg of plasmid in 1 h at 37°C. Use of high-specific activity topoisomerase I preparations is essential to avoid perturbation of the linking-number distribution by inactive enzyme molecules that may bind to DNA.
4. Relaxation with wheat-germ topoisomerase I can be carried out at any temperature from 15°C to approximately 40°C.
5. A common problem is that the topoisomer distributions do not overlap enough to give a quasiuniform distribution of topoisomers when mixed. This is why it is important to analyze the individual equilibrium distributions before pooling the reactions. If there is insufficient overlap, additional reactions containing intermediate concentrations of intercalator should be prepared.
6. This protocol was originally developed using T2 topoisomerase, but should also apply to other type-II enzymes, including those from human cells.

7. Although any horizontal gel electrophoresis apparatus can be used, we prefer the Onephorall electrophoresis units from IBI-Shelton Scientific. These electrophoresis tanks are designed to recirculate buffer with the use of a magnetic stirrer and are therefore well suited for analyzing DNA topology.

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