Type II topoisomerases change DNA topology by passage of one DNA duplex (the transfer, T-segment) through a transient double-stranded break in another (the gate, G-segment). Here we monitor the passage between short double-stranded DNA segments within long single-stranded DNA circles that leads to catenation of the circles. To facilitate catenation, the circles were brought into close proximity using a tethering oligonucleotide, which was removed after the reaction was complete. We varied the length and the composition of the reacting DNA segments. The minimal DNA duplex length at which we detected catenation was 50–60 bp for DNA gyrase and 40 bp for topoisomerase IV (Topo IV). For Topo IV, catenation was observed when one, but not both, of the DNA-DNA duplexes was replaced by a DNA-RNA duplex. Topo IV cleaved the DNA-DNA duplex, but not the DNA-RNA duplex implying that the DNA-RNA duplex can be a T-segment but not a G-segment.

DNA topoisomerases alter DNA topology and thereby contribute in vivo to DNA structure and metabolism (1–3). Their most prominent biological role is the removal of DNA entanglements and superhelical stress during DNA replication and sister-chromatid separation (4–8). All topoisomerases act via the creation of transient breaks in DNA. In the case of type I topoisomerases, these breaks are single-stranded; in the case of type II topoisomerases they are double-stranded. Type II topoisomerases act by passing one DNA segment (the transfer- or T-segment) through the break that they transiently introduce in another DNA segment (the gate- or G-segment) (9). Depending on the DNA substrate and the reaction conditions, type II topoisomerases can catalyze DNA catenation/decatenation, knotting/unknotting and introduction and removal of DNA superturns.

As type II topoisomerases act on duplex DNA, two questions arise: what is the minimal length of the DNA substrate and what is the enzyme specificity for the double-stranded DNA. In additional to providing information about the DNA-enzyme interaction, these questions might have direct biological relevance because topoisomerases could encounter, while assisting replication, single-stranded DNA, DNA-RNA duplexes, and DNA that is partially coated by other proteins so that only short regions could be available for topoisomerase action. The interaction of type II topoisomerases with very short substrates is most commonly investigated by the DNA cleavage assay, which is based on trapping of the covalent complex between the enzyme and the transiently cleaved G-segment by certain chemicals, such as quinolones (10, 11). Upon enzyme denaturation, DNA breaks are produced, which can be readily detected by electrophoresis. Cleavage showed, for example, that gyrase acts with some sequence specificity (12–14) and that the minimal cleavable substrate is about 20–25-bp long for DNA gyrase (15, 16) and for eukaryotic type II topoisomerases (17–19). It has been also shown that substrates other than pure DNA duplexes are recognized by eukaryotic type II topoisomerase. For example, the presence of a few ribonucleotides at certain positions within DNA duplexes increases type II topoisomerase cleavage several times, though full substitution of one strand by RNA completely inhibits topoisomerase-mediated cleavage (20, 21). Interestingly, eukaryotic topoisomerase II is able to cleave parallel DNA tetraplexes at the tetraplex/ssDNA junction (22). However, the disadvantage of the cleavage assay is that it detects only part of the enzymatic cycle. Another approach is based on the reaction of the enzyme with small closed double-stranded DNA circles. Circles as short as 174 bp can be negatively supercoiled, and positively supercoiled circles as short as 116 bp can be relaxed by DNA gyrase (23). However, using this approach it is difficult to test shorter substrates, because they would not readily circularize. Therefore, we developed a system that allows the investigation of the complete duplex passage reaction for short substrates. We applied it mainly to the study of the duplex passage reaction by Escherichia coli gyrase and topoisomerase IV (Topo IV)2, but it can be used for other type II topoisomerases.

**MATERIALS AND METHODS**

DNA—Long single-stranded DNA circles designated BC (3.4 kb) and BS (3 kb) were obtained from phagemids pBC SK(+) and pBluescript SK(+) (Stratagene), respectively, using the Stratagene protocol for circular single-stranded DNA.

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phagemid DNA recovery and gel-purified from a 1% agarose gel. Oligonucleotides were purchased from Integrated DNA Technologies, except MCS100, which was purchased from Operon.

Substrates for Catenation Reaction
—Hybridization between the single-stranded (ss) DNA circles (20 nM), tethering oligo (7 nM) and substrate oligo (73 nM) was performed in 10 mM MgCl₂ and 100 mM Tris-HCl (pH 7.6). The mixture was successively incubated at 72 °C for 6 min, 58 °C for 40 min, and 37 °C for 3 h.

Enzymes—GyrA and GyrB subunits of E. coli gyrase were purified by Allyn Schoeffler from the James Berger laboratory (University of California, Berkeley) as described (34).

The ParC and ParE subunits of E. coli topoisomerase IV (Topo IV) were purified by Nancy Crisona and Brian Peter from the Nicholas Cozzarelli laboratory (University of California, Berkeley) as described (44).

Enzymatic Reactions—Catenation reaction was performed in a volume of 10 μl with a total concentration of the single-stranded DNA circles of 4 nM. Enzyme concentration in the reaction mixture was 50 nM in the case of gyrase, and 10 nM in the case of Topo IV. The reaction buffers were, for gyrase: 25 mM Tris-HCl (pH 7.6), 40 mM KCl, 2 mM MgCl₂, 1 mM spermidine, 0.5 mM ATP, 1.2 mM dithiothreitol, 4% glycerol, 50 μg/ml bovine serum albumin; for Topo IV: 100 mM potassium glutamate, 25 mM Tris-HCl, pH 7.6, 10 mM MgCl₂, 1 mM ATP, 0.5 mM dithiothreitol, 5% glycerol, 50 μg/ml bovine serum albumin. The samples were incubated at 32 °C for 1 h. The reaction was stopped by the addition of 2.4 μl of 5× stop buffer (25 mM EDTA, 2.5% SDS) in the case of gyrase, or 1 μl of 250 mM EDTA followed by 2.4 μl of 5× stop buffer in the case of Topo IV. The mixture was supplemented with 0.5 μl of proteinase K (20 mg/ml) and incubated at 37 °C for 0.5 h. Cleavage assays were performed essentially as described in Ref. 15.

Displacement of Tethering Oligos—The displacing oligo was added up to 1.4 μM. Samples were incubated 6 min at 73 °C, 1 h at 58 °C, and cooled down to room temperature. After addition of an equivalent volume of 0.3 M NaOH, the samples were incubated at room temperature for 5 min, supplemented with 2 μl of the loading buffer (30% glycerol, 3.5 mg/ml bromphenol blue, and xylene cyanol in TAE 1×), and loaded on an agarose gel. Alternatively, the samples were additionally heated for 6 min at 73 °C before loading without NaOH addition.

**Gel Electrophoresis and Southern Blot**—Native gel electrophoresis was performed in 1.2% agarose in TAE 1× buffer at 3.5 V/cm for 14 h. The DNA was detected by Southern blotting.
Alkaline agarose gel electrophoresis was performed as described in Ref. 45.

**Data Analysis**

The apparent yield of catenanes (C) was calculated using Equation 1,

\[ C = \frac{100\% \times (\text{PE}^+ - \text{PE}^-)}{P_0} \]  

(Eq. 1)

where \( P_0 \) is the initial percentage of the tethered circles, *i.e.* without displacement and topoisomerase treatment, \( \text{PE}^+ \) is the percentage of the tethered circles treated by topoisomerase that remains after displacement of the tethering oligo, and \( \text{PE}^- \) is the percentage of the tethered circles not treated by topoisomerase that remains after displacement of the tethering oligo. \( \text{PE}^- \) represents the background amount of dimeric species due to non-complete displacement of the tethering oligo or because of some trace amount of dimeric single-stranded circles remaining after gel purification.

**RESULTS**

**Experimental Strategy**

The general strategy used in our experiments is based on the topoisomerase-mediated duplex passage between short double-stranded regions within long single-stranded DNA circles (Fig. 1A). These short double-stranded regions (substrate duplexes) were created by hybridization of the substrate-forming oligonucleotides (oligos) (red) to the single-stranded circles. Duplex passage leads to catenation of the DNA circles, which contain the duplexes. To facilitate the reaction between the two substrate duplexes, they are brought together by a tethering oligo (blue). The tethering oligo contains two short regions (21–25 nt), which hybridize to the single-stranded circle close to the substrate duplex (at about 30 nt), and a oligothymine linker between them (dT3–10, see inset in Fig. 1A). After topoisomerase-mediated duplex-transfer between substrate duplexes, the tethering oligo can be removed.
(displaced) by heating in the presence of a displacing oligo (Table 1). After displacement, only catenated circles remain together, as a consequence of duplex passage.

**Substrates**—Two long single-stranded DNA circles BC (3.4 kb) and BS (3 kb) were used to create various tethered circular substrates (Fig. 1B). Within these circles, three regions were used for hybridization with substrate-forming and tethering oligos: 1) the multiple cloning site (MCS) region, which is the same both for BC and BS, 2) the ampicillin (Amp) gene region, which is present only in BS, and 3) the chloramphenicol (Cam) gene region, which is present only in BC.

All substrate-forming and tethering oligos are shown in Tables 1 and 2. The 3′-end of the substrate-forming oligo is proximal to the tethering oligo. All tethering oligos, except the Cam-Amp tether, bind two identical regions within two single-stranded circles, and the two substrate duplexes are also identical in this case. We call the tethered circles created by these tethering oligos the “symmetric system.” Cam-Amp tether creates an “asymmetric system” (shown in Fig. 1B, *box*), where the two substrate duplexes are different from each other, and can be varied independently. Unless it is otherwise indicated, in all tethered circles the substrate-forming and tethering oligos hybridize within the same region of the circle (for example, the MCS substrate-forming oligos are used with the MCS tether).

### Catenation Catalyzed by DNA Gyrase

**Both Gyrase Subunits Are Required for Catenation**—DNA gyrase is heterotetramer containing two subunits, called GyrA and GyrB (24–27). The GyrA and GyrB subunits are purified separately and mixed together before the reaction to reconstitute the enzyme (A₂B₂). A typical result of the catenation reaction is shown in Fig. 2. The left panel shows the tethered circles before displacement (*i.e.* when the circles are held together by the tethering oligo regardless of catenation). As expected, the amount of the non-displaced tethered circles is the same in the presence and in the absence of the enzyme (*lanes 1–7*). The picture changes dramatically after displacement of the tethering oligo (*lanes 8–14*). Only in the presence of both gyrase subunits does a significant amount of tethered species survive (*lanes 8, 11, and 12*). The requirement for both subunits for catenation provides a control that the reaction is catalyzed by gyrase rather than by some enzymatic contamination in either of the subunits.

**Restriction Analysis of the Reaction Products**

The MCS100 substrate-forming oligo was used in these experiments. Only reaction products that remain after displacement are shown. In *lanes 1–4*, pBC is in the ScaI-cleavable form. In *lanes 7–10*, pBS is in the ScaI-cleavable form. The cleavable ScaI-site is shown by a dot on the circle.
gyrase action on tethered circles are really catenanes, we performed experiments with mixed BC- and BS-tethered circles, where either the BC or BS circle were cleaved by a restriction enzyme, Scal. To convert either circle into a Scal-cleavable form, it is hybridize with its corresponding Sca-oligo (Table 2). Because both BC and BS circles have common MCS regions, three possible combinations of tethered circles, BC/BC, BC/BS, and BS/BS are formed upon hybridization of the mixture of BC and BS circles with substrate-forming and tethering oligos complementary to the MCS region (Fig. 3, lanes 2 and 8). As seen in Fig. 3, gyrase-treated BC/BS-tethered circles, which contain only one circle in the Scal-cleavable form, disappear upon Scal cleavage and subsequent tethering oligo displacement, and no new dimeric species appear, which is consistent with catenanes (lanes 1 and 7).

An additional confirmation that the observed products are catenanes comes from the fact that they survive gel electrophoresis under denaturing (alkaline) conditions (supplemental Fig. 1). It is interesting that under these electrophoretic conditions the catenanes resolve into several bands, which are most likely catenanes with multiple links because of several successive duplex passage reactions.

The Catenation Reaction Requires ATP—Fig. 4 shows the result of catenation reactions with and without ATP for the same tethered circles as in Fig. 2. The yield of the reaction in the presence of 0.5 mM ATP is 11%, whereas in the absence of ATP it is 0.6%, close to the background level of our method (about 0.5%).

The Reaction Occurs Only between Proximal Regions of the Circles—In our method the tethering oligo specifically brings together those regions of the circles that are close to the tethering oligo binding site. We will refer to these regions as "proximal," and the regions roughly on the opposite side of the circles will be referred as "distal." Both proximal and distal regions can be converted to DNA duplexes by hybridization to corresponding substrate-forming oligos.

We thus investigated whether the two duplex regions must be proximal for catenation to occur. We used two tethering oligos, the MCS-tether and Amp-tether, which hybridize to the BS circle within the MCS and Amp-regions, respectively, and two substrate oligos, MCS80 and Amp100, which hybridize to the BS circle in the proximity (32 nt) of the corresponding tethering oligo. The hybridization sites for the MCS and Amp oligos are on the opposite sides of the BS circles, roughly 1.5 kb from each other. Consequently, in the tethered circles with the Amp tether and the MCS substrate oligo, and vice versa, substrate duplexes are distal from each other. With the Amp tether and the Amp substrate oligo, and with the MCS tether and the MCS substrate oligo, substrate duplexes are proximal (schematized in Fig. 5, top). Fig. 5 shows that only proximal combinations produce a significant yield of catenanes (lanes 1 and 7). This result also implies that there is no detectable duplex-passage reaction between the 25 bp duplexes formed by the tethering oligo itself, probably because they are too short and too close.

Under our reaction conditions we detected catenanes only for tethered circles, although at five times higher spermidine concentration (5 mM), which facilitates DNA aggregation (28), we observed, without a tether, a low yield of catenanes (around 1%, data not shown). In contrast, with double-stranded plasmid DNA, more than 50% catenation is observed with DNA gyrase at this high spermidine concentration (28–30). A probable explanation for this difference is that in the case of completely double-stranded plasmids any randomly chosen regions, which are brought together by aggregation, could be suitable for duplex passage. In our case, however, only about 4% of the DNA within tethered circles is double-stranded. Thus, the probability that these double-stranded regions are close to each other within the aggregate is small.

Duplex Length Dependence for the Catenation Reaction—We investigated the length dependence of catenation using substrate-forming oligos of different length. First, we investigated the symmetrical system, in the MCS region, where both duplexes are the same. The data are summarized in Fig. 6A. The apparent yields of catenanes for different substrate oligo
Topoisomerase Action on Short Duplexes

lengths are normalized to the yield for the maximal length, *i.e.* MCS100.

The yield of the reaction increases with the duplex length, from 50 bp to 80 bp. The yield in the case of the 50 mer is at least ten times less than in the case of the 80 mer, at the limit of sensitivity of our method (about 1% or less). Because this effect is significantly larger than the $1.6 \times$ decrease in length from 80 bp to 50 bp, it cannot be explained by the simple decrease in the number of landing sites for the enzyme.

We next showed that the observed length dependence is not caused by the absence of some specific sequence in the shorter oligos (such as MCS50). To explore this possibility, we mapped predominant gyrase cleavage sites within the MCS100 substrate duplex. We found five cleavage sites of different intensities (supplemental Fig. 2A). Three of these sites are localized within the MCS80 and MCS67 sequence, and two of them are within the MCS50 sequence. Similar results were obtained for the Cam and Amp duplex regions (supplemental Fig. 2B and C), indicating that gyrase-mediated cleavage sites are present even in the shorter duplexes. Further increase in the substrate duplex length (from 80 bp to 100 bp) produces some decrease in the efficiency of catenation (see “Discussion”).

In the symmetrical system, we can only change the length of both segments simultaneously. However, because G- and T-segments play different roles and are interacting with different parts of the enzyme, the length requirements for G- and T-segments could be different. These questions were investigated using the asymmetric system. Fig. 6B shows the yields of catenation where the longest duplex regions (either Amp100 or Cam100) remain constant, while the length of the other duplex varies. In either case, shortening of the duplex by only 2-fold, from 100 bp to 50 bp, decreased the yield of the tethered circles 5-fold in one case, and about 20-fold in the other. When the varied duplex was 21 bp, catenanes could not be detected in either case. Thus, at least 50 bp participate in the interaction with the enzyme for both the G- and T-segments. Importantly, single-stranded DNA cannot serve as a G- or a T-segment.

Catenation Reaction Catalyzed by Topo IV

To check if our method could be used with other enzymes, we applied it to another type II topo, *E. coli* Topo IV (31). Topo IV is also a heterotetramer. It contains two subunits, ParC and ParE, analogs of GyrA and GyrB, respectively. The major difference between these two enzymes is that Topo IV is not able to induce negative supercoiling in DNA, but is much more efficient in unknotting and decatenation (32–35). Thus, Topo IV
and DNA gyrase share significant structural similarity, but strongly differ in their DNA substrate preferences and predominant mode of action. As in the case of gyrase, the reaction required both Topo IV subunits (Fig. 7A, lane 1) and the presence of ATP (Fig. 7B, lane 1). The yield of catenanes increased with the duplex length (Fig. 7C), and catenation was not detected without the tethering oligo (data not shown).

Topo IV Can Use a DNA-RNA Duplex, Probably as a T-segment—As in the case of gyrase, both duplexes were necessary for detectable Topo IV-induced catenation (Fig. 7D). However, Topo IV-induced catenation was also observed when either one (Fig. 8A, lanes 3 and 5), but not two (lane 7), of the DNA-DNA substrate duplexes was replaced by a DNA-RNA duplex. Fig. 8B shows Topo IV-induced cleavage of the substrates containing DNA-DNA and DNA-RNA duplexes in the presence of norfloxacin to stabilize the cleavage products (36). RNA- and DNA-containing asymmetric tethered circles used in this experiment were the same as in Fig. 8A. The Amp50 oligo is complementary to the BS circle, and the Cam50 oligo is complementary to the BC circle. Linearization of the corresponding plasmid, which indicates cleavage, is observed only for the plasmid when it is hybridized to the DNA oligo, but not to the RNA oligo (Fig. 8B, lanes 3 and 5 for Amp50, 9 and 11 for Cam50). Thus, we conclude that DNA-RNA duplexes can serve as T-segments, but not as G-segments.

The yield of catenanes was always lower when one duplex was a DNA-RNA duplex in comparison with both DNA-DNA duplexes (compare lanes 3 and 5 to lane 1, Fig. 8A), though this difference was smaller when a 100-bp long DNA-DNA G-segment was used instead of a 50-bp long DNA-DNA G-segment (supplemental Fig. 3A). In the case of DNA gyrase, we were not able to detect catenation when one of the duplexes was DNA-RNA, using the same substrates as for Topo IV (supplemental Fig. 3B). However, since the efficiency of catenation in general is lower for gyrase in comparison with Topo IV, it could be that the catenation is below the sensitivity of our method in this case.

DISCUSSION

In this work we constructed and validated an experimental system to investigate the duplex passage reaction catalyzed by type II topoisomerases. The substrate duplexes are localized within long single-stranded DNA circles, and the duplex passage reaction leads to the formation of catenanes between the circles, which can be identified unambiguously. The catenation occurs only if substrate duplexes within the circle are brought close to each other by a tethering oligo. Thus, our system allows the targeting of the duplex passage reaction to any given sequence within a single-stranded circle by annealing an appropriate tethering oligo. The system can be used to investigate sequences, which affect topoisomerase activity, for example the strong gyrase site in Mu-phage (14).

We studied the catenation reaction for two E. coli type II DNA topoisomerases: gyrase and Topo IV. The efficiency of the catenation reaction for DNA gyrase in our system grew strongly.
with the length of the duplex from 50 to 80 bp. Judging from footprinting experiments, within a long double-stranded DNA substrate, the length of the DNA involved in the complex with gyrase is about 140 bp. However, within these 140 bp, 40–50 bp in the vicinity of the gyrase cleavage site were protected more strongly than the rest of the sequence (10, 11, 37, 38). This suggests that perhaps only these 40–50 base pairs are essential for the gyrase-mediated duplex passage reaction itself and that the rest of the DNA (about 100 bp), involved in the complex with gyrase, wraps around the C-terminal domain of GyrA subunit (GyrA CTD). The DNA wrapping around the GyrA CTD is necessary for generation of negative supercoiling (39–42). However, the role of GyrA CTD in the reactions between remote DNA segments, as in catenation, is less clear. The fact that in our system the maximal yield of catenanes is obtained when the substrate duplex is significantly longer than the length of the strongly protected DNA area in the vicinity of the gyrase catalytic site (i.e. 80 bp versus 40–50 bp) might suggest that the CTD somehow facilitates the catenation reaction in our system, for example by stabilizing the complex with the G-segment, or by interacting with the T-segment, as suggested for the ParC CTD of Topo IV (34). However, when the duplex regions become too long, a partial wrapping of the G-segment around the enzyme could compete with the interaction of the enzyme with the intermolecular T-segment, explaining why for DNA gyrase we observe a slight decrease in catenation when the length of the duplex increases above 80 bp. The better understanding of this effect requires further investigation.

By using an asymmetric system, we can investigate the reaction between two DNA segments with different lengths or backbone composition. The fate of the two DNA segments that participate in the duplex passage reaction is quite different: the G-segment undergoes cleavage and re-ligation, while the T-segment remains intact. Thus, G- and T-segments may have different requirements for nucleic acid length and composition. We showed that for gyrase- and Topo IV-mediated duplex passage both DNA segments participating in the reaction must be double-stranded, and decreasing the length of either duplex strongly decreases the yield of catenanes. Thus, the double-stranded DNA structure is required not only for cleavage of the G-segment, but also for interaction with the T-segment. We also showed that Topo IV can catalyze the catenation reaction when one (but not both) DNA-DNA duplex was replaced by a DNA-RNA duplex. Cleavage experiments in the presence of norfloxacin show that Topo IV cleaves a DNA-DNA, but not a DNA-RNA duplex, suggesting that the DNA-RNA duplex works as a T-segment but not as a G-segment. By performing similar experiments using various DNA analogs with modified backbones it would be possible to distinguish which DNA chemical groups are important for the DNA-gyrase interaction within both the T- and G-segments. This is especially useful for the interactions with the T-segment, which cannot be monitored by a DNA cleavage assay, as is the case of the G-segment.

The experimental system developed in this work is applicable to other types of topoisomerases. Preliminary data with E. coli Topo IA showed that it produces catenanes in our system (data not shown). E. coli Topo IA (as well as other type IA topoisomerases) catalyzes a single-strand DNA passage reaction similar to the duplex passage reaction of type II topoisomerases (29, 43). In our system, Topo IA presumably acts on single-stranded or partially double-stranded regions of the circles proximal to the tethering oligo. By varying these regions, one could investigate, for example, how Topo IA activity varies with the sequence in both DNA strands or with the presence of the double-stranded regions.

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