Transcription-dependent recombination induced by triple-helix formation

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The homologous recombination between direct repeat sequences separated by either 200 or 1000 bp was induced by active transcription of the downstream gene when poly(dG)–poly(dC) sequences exist between the two direct repeats. This dG tract-mediated and transcription-induced recombination was RecA independent, and the frequency of recombination was dependent on both the length and the orientation of the poly(dG)–poly(dC) sequences relative to the gene. An intramolecular dG.dG.dC triplex formation was detected in *Escherichia coli* cells in a length-dependent manner when the transcription of the downstream gene was activated. We suggest that the negative superhelical strain generated by active transcription of the downstream gene induces poly(dG)–poly(dC) sequences to adopt a triple-helix structure in vivo and that this structure brings two remote sequences together to stimulate homologous recombination.

[**Key Words:** recombination; transcription; negative supercoiling; DNA structure; triple helix]

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The factors that influence the degree of supercoiling of a DNA inside a bacterium include transcription, diffusional pathways, gyrase-catalyzed negative supercoiling, and DNA topoisomerase I-catalyzed relaxation of negatively supercoiled DNA (Liu and Wang 1987; Wu et al. 1988). It was first proposed by Liu and Wang (1987) that active transcription may generate a localized positive supercoiled domain and a negative supercoiled domain in front of and behind a transcribing RNA polymerase, respectively. This is based on the concept that the DNA being transcribed may turn around its helical axis instead of the elongating RNA polymerase molecule rotating around the DNA. This twin-supercoiled-domain model has now been supported by considerable evidence from in vitro and in vivo studies (Brill and Sternglanz 1988; Giaever and Wang 1988; Tsao et al. 1989). Because bacterial gyrase and topoisomerase I act differently on positively and negatively supercoiled DNA, in vivo studies for bacteria and yeast have made use of topoisomerase mutants in testing the twin-supercoiled-domain model (Brill and Sternglanz 1988; Giaever and Wang 1988).

Certain DNA sequences adopt non-B DNA structures when subjected to negative superhelical strain. These sequences could be used to probe local superhelical strain inside cells. Previously, we placed one of these sequences, a poly(dG)–poly(dC) sequence 5' of a reporter gene and examined its effects on transcription in mammalian cells. If supercoiled strain was in fact generated by the process of transcription, this sequence would readily adopt an intramolecular dG.dG.dC triplex struc-

ture and might exert effects on the level of transcription. Our results suggest that this sequence augments transcriptional activity of the gene when it is in the double-stranded form but not when it is forming a triplex (Kohwi and Kohwi-Shigematsu 1991). In *Escherichia coli* cells, an intramolecular dG.dG.dC triplex formation was directly demonstrated by chemical probing. This triplex formation was detected in chloramphenicol-treated cells within which there was a higher degree of supercoiling (Kohwi et al. 1992).

Non-B-form DNA structure that would be stabilized by local supercoiling generated by the process of active transcription may have significant effects not only on transcription but also on recombination. Recent work in the site-specific recombination of λ phage within the *E. coli* system shows that bending of the phage attachment site, *attP*, induced by integration host factor (IHF) and superhelical strain exerted on this DNA, contribute to the formation of the active nucleoprotein assembly unit, the intasome (for review, see Landy 1989; Nash 1990). DNA sequences that have the potential to bend can successfully substitute for the *attP* site and facilitate site-specific recombination (Goodman and Nash 1989). We decided to test whether non-B DNA structure that would be stabilized by the process of transcription has a significant effect on homologous recombination in an *E. coli* system.

In the study of homologous recombination, much attention has been given to the steps of DNA strand pairing and the exchange of homologous sequences in which RecA protein plays a central role (for review, see Radding 1988; Smith 1988; Kowalczykowski 1991). It still remains uncertain, however, as to how two homologous

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sequences find each other in the first place to stimulate recombination. In searching for a clue to answer this question, it is of interest that there is considerable evidence for stimulation of recombination by transcription. Correlation between recombination and transcription has been well-documented in the yeast system. A DNA segment containing an RNA polymerase I promoter, *HOT1*, stimulates recombination of an adjacent gene by polymerase I transcription initiating in the inserted rDNA and proceeding through the adjacent sequence (Voelkel-Meiman et al. 1987). Also in yeast, RNA polymerase II-dependent transcription stimulates direct repeat recombination leading to loss of a plasmid integrated at the *GAL10* locus (Thomas and Rothstein 1989 a,b). In the prokaryotic system, there is also a report suggesting that transcription stimulates recombination (Dul and Drexler 1988). In higher eukaryotes, correlation between transcription and recombination has been suggested on the basis of the fact that transcription of the immunoglobulin variable (V) region genes occurs at a time when the cells are undergoing a B cell-specific V_H-D/J_H recombination event (Blackwell et al. 1986; Yancopoulos et al. 1986). It is not certain how transcription stimulates recombination, but it is possible that transcriptionally active chromatin structures make the templates more accessible to both RNA polymerases and recombinases. Alternatively, one could imagine that local supercoiling generated by the process of active transcription induces formation of altered DNA structures and that this subsequently contributes to stimulation of recombination.

In this study we employed poly(dG)–poly(dC) sequences as model sequences to examine the potential role of non-B DNA structures in homologous recombination. Here, we show that the process of active transcription of a downstream gene stabilizes the formation of an intramolecular dG.dG.dC triplex and promotes the homologous recombination between direct repeats in a RecA-independent manner.

Results

Transcription-dependent recombination assay system

To examine recombination events under negative superhelical strain induced by transcriptional activation, we employed a strong, isopropyl- β -D-thiogalactopyranoside (IPTG)-inducible bacterial promoter, the *tac* promoter (Ptac), (Fig. 1). The pGEX-2T vector (Pharmacia) has one copy of the *lac* promoter (Plac), which is IPTG inducible but a weaker promoter, in addition to the Ptac promoter. To this vector, one additional copy of the *lac* promoter (Plac) was introduced upstream of the Ptac promoter. Both Ptac and Plac share homologous sequences that differ only by 2 bases at positions –8 and –9 from the transcriptional start site (+1), sharing the rest of the 53-base sequences (see Fig. 3A, below). In the final construct, there are a total of three homologous, directly repeated sequences as potential sites for recombination. These homologous sequences found in Plac and Ptac pro-

Transcription-induced triplex and recombination

Figure 1. Construction of plasmids for transcription-driven recombination assay. The plasmid pL-GEX was constructed from pGEX-2T (Pharmacia) by introducing a *PvuII*–*EcoRV* fragment of the *lac* promoter (Plac) of Bluescript (Stratagene) as the second Plac at the *SspI* site of pGEX-2T. The resulting plasmid pL-GEX consists of two Plac and one Ptac promoters in the same direction. *EcoRI*–*BamHI* fragments containing various lengths of poly(dG)–poly(dC) sequences from pCAT[C/G]_n were introduced at the *XhoI* site of pL-GEX, almost at the center of 170 bp between the second Plac and the Ptac. The poly(dG) sequence on the top strand (the coding strand of the GST gene) was named as pL-GEX-G. The poly(dC) sequence on the top strand was named as pL-GEX-C. P1, P2, and P3 represent the identical sequences of 5'–*lac* promoter (5' Plac), 3'–*lac* promoter (3' Plac) and *tac* promoter (Ptac), respectively (see Fig. 3). The map is not drawn to scale.

moters are designated as P1 (in 5'–Plac), P2 (in 3'–Plac), and P3 (in Ptac) as shown in Figure 1. P1 and P2 are 800 bp apart, and P2 and P3 are 200 bp apart. In addition, varying lengths of poly(dG)–poly(dC) sequences were inserted at the unique *XhoI* site between P2 and P3. The resulting construct, the pL-GEX-C/G plasmid [pL-GEX-C_n or pL-GEX-G_n, depending on whether *n* number of residues of poly(dC) or poly(dG) sequences are on the coding strand], was employed to test transcription-dependent recombination.

On the basis of the twin-domain model (Liu and Wang 1987), the dG tracts are expected to experience negative superhelical strain after IPTG induction because Ptac is a much stronger promoter than Plac [Ptac is estimated to be 100 times stronger in promoter activity than Plac on the basis of the two different assay systems (deBoer et al. 1983; Borowiec and Gralla 1987)]. The recombination events between the homologous sequences in the Ptac and Plac promoter regions that occurred as a consequence of transcriptional activation induced by IPTG and the presence of the triplex forming poly(dG)–poly(dC) sequences upstream of Ptac were examined. The original pGEX-2T vector contains the overexpressed *lacI*^q allele of the *lac* repressor; therefore, expression of

glutathione *S*-transferase (GST) proteins from the strong *tac* promoter is efficiently repressed until induction with IPTG, regardless of the *lacI* status of the *E. coli* host (Smith and Johnson 1988). We confirmed that this was also true for both the pL-GEX plasmid and pL-GEX containing poly(dG)-poly(dC) sequences: GST protein was made only after IPTG induction, and no significant difference was detected in the level of GST protein expression between pL-GEX and pL-GEX containing poly(dG)-poly(dC) sequences (data not shown).

Effects of IPTG-induced transcription on recombination events in *recA*[−] and *recA*⁺ cells

To test the effect of transcription on recombination events in these constructs, plasmid DNAs (pL-GEX, pL-GEX-G₂₁, and pL-GEX-C₂₇) were transfected into *E. coli* AB1157 (*recA*⁺) and AB2463 (*recA*[−])-competent cells and cultured on amp-LB agar plates with or without IPTG (0.5 mM) at 37°C for 16 hr. AB2463 (*recA*[−]) cells have the same genetic background as AB1157 (*recA*⁺), (Howard-Flanders and Theriot 1966). To prepare plasmid DNAs, 60 colonies from agar plates were inoculated into amp-LB broth and cultured in a shaker at 37°C for 16 hr without IPTG. The additional 16-hr outgrowth of the culture does not result in selection in our system as described in detail in Results (see Fig. 4, below). The *EcoRV*–*EcoNI*-digested plasmid DNAs were separated by 1% agarose gel electrophoresis and blotted to a nylon membrane (Zeta-probe, Bio-Rad). The membrane was hybridized with the ³²P-labeled pL-GEX plasmid DNA. As shown in Figure 2, without IPTG induction (no transcriptional activation on *Ptac* and *Plac*), all constructs showed the original size of *EcoRV*–*EcoNI* fragments [Fig. 2, IPTG (−), lanes 2,4,6,8,10,12; 1400 bp indicated by b]. On the other hand, after IPTG induction for 16 hr on an agar plate, the control plasmid, pL-GEX without dG tracts, showed shorter *EcoRV*–*EcoNI* fragments in *recA*⁺ cells [Fig. 2, IPTG (+), lanes 3; 1200 bp indicated by c] but not in *recA*[−] cells [Fig. 2, IPTG (+), lane 5]. The plasmid constructs with poly(dG)–poly(dC) sequence insertions, pL-GEX-C₂₇ and pL-GEX-G₂₁, showed shorter fragments not only in *recA*⁺ cells but also in *recA*[−] cells upon IPTG induction (Fig. 2, lanes 7,9,11,13; 1200 bp indicated by c). For pL-GEX-G₂₁, even shorter fragments of 400 bp were detected as well (Fig. 2, lanes 11,13; 400 bp indicated by d). We call the deletion that led to the larger fragment of 1200 bp, type I recombination, and the shorter fragment of 400 bp, type II recombination. The results of transcriptional activation of a bacterial gene by IPTG show two important facts: A specific deletion mutation in the control pL-GEX plasmid occurs in a RecA dependent manner, and insertion of poly(dG)–poly(dC) sequences into the upstream region of the gene results in specific deletion mutations in a RecA independent manner. In this paper we focused on the effects of poly(dG)–poly(dC) sequences on homologous recombination in response to transcriptional activation. The mechanism for RecA dependent, transcription-induced recombination

Figure 2. Detection of transcription-dependent recombination in plasmids. A 16-hr IPTG induction to activate transcription was performed on *E. coli* AB1157 (*recA*⁺) and AB2463 (*recA*[−]) cells bearing plasmids pL-GEX, pL-GEX-C₂₇, and pL-GEX-G₂₁ on amp-LB agar plates. The transfected cells were plated on amp-LB agar plates with or without 0.5 mM IPTG. The plasmid DNAs were prepared from an overnight culture of 60 pooled colonies in amp-LB medium without IPTG. The *EcoNI*–*EcoRV* fragments were analyzed by Southern blot analysis. The blotted membrane was hybridized with ³²P-labeled plasmid DNA, pL-GEX. Restriction enzyme digestion patterns of pL-GEX, pL-GEX-C₂₇, and pL-GEX-G₂₁ plasmid DNAs are shown with (+) and without (−) IPTG induction. (Lane 1) λ HindIII DNA size marker; (lanes 2–5) pL-GEX; (lanes 6–9) pL-GEX-C₂₇; (lanes 10–13) pL-GEX-G₂₁; (lanes 2,4,6,8,10,12) IPTG(−); (lanes 3,5,7,9,11,13) IPTG(+); (lanes 2,3,6,7,10,11) *recA*⁺; (lanes 4,5,8,9,12,13) *recA*[−]. (a) *EcoRV*–*EcoNI* 3800-bp fragment; (b) *EcoRV*–*EcoNI* 1400-bp fragment; (c) *EcoRV*–*EcoNI* 1200-bp fragment; (d) *EcoRV*–*EcoNI* 400-bp fragment.

observed for the control pL-GEX plasmid deserves independent study and is currently under investigation.

Identification of type I and type II recombination sites

The recombination sites were analyzed by direct sequencing of the DNA fragments (*EcoRV*–*EcoNI*, 3′-labeled at the *EcoNI* site) generated by type I and II recombination. We also selected 10 colonies from IPTG-induced *E. coli* carrying pL-GEX-C₂₇ or pL-GEX-G₂₁ to obtain type I and type II recombinants. The nucleotide sequence of pL-GEX from *EcoRV* to *EcoNI* sites is shown in Figure 3A. The poly(dG)–poly(dC) sequences were inserted into the *XhoI* site. The sequence analysis of type I recombination (1200 bp of the *EcoRV*–*EcoNI* fragment) revealed that the sequence between P2 and P3, including poly(dG)–poly(dC) or the sequence between position 1177 and 1365 was deleted. The sequence of type II recombination (400 bp of the *EcoRV*–*EcoNI* fragment) showed that the sequence between P1 and P3, including poly(dG)–poly(dC), or the sequence between position 403 and 1365 was deleted. The results indicate that the type

Figure 3. Analysis of recombination sites. (A) Sequences of recombination sites: The nucleotide sequence of the *EcoRV*–*EcoNI* fragment of pL-GEX is shown. The nucleotide numbering starts at the 5' end of the *EcoRV*-cleaved sequence. The *EcoNI* site is located 1401 bp away from the *EcoRV* site, excluding dG tracts. The identical sequences between P1 and P2 regions are underlined with uppercase letters and correspond to solid boxes in B. The identical sequences among P1, P2, and P3 regions are open boxed with boldface letters and correspond to open boxes in B. Poly(dG)–poly(dC) sequences were introduced in the *XhoI* site as described in Materials and methods. The +1 site (the transcriptional start site) of each promoter was indicated. Double-headed open arrows (I) indicate type I recombination region between P2 and P3. Double-headed closed arrows (II) indicate type II recombination region between P1 and P3. (B) Recombination products after transcriptional activation: Plasmid sequences of pL-GEX-C/G are represented by horizontal rules. The transcription was activated by addition of IPTG and is depicted by an arrow. The analysis of plasmids after transcriptional activation resulted in two types of recombination products (type I and type II). (RV) *EcoRV*; (PvuII) *PvuII*; (Ssp) *SspI*; (N) *EcoNI*; (dG/dC) poly(dG)–poly(dC) sequence; (bp) base pairs.

I recombination (200 bp deletion) occurred at the identical direct repeat sequences between position 1134 and 1176 in P2 (bold letters) and position 1323–1365 in P3 (bold letters) deleting the dG tract and the *Ptac* promoter. The type II recombination (1000 bp deletion) occurred at the same sequences between position 360–402 in P1 (bold letters) and position 1323–1365 in P3 (bold letters)

deleting all of the dG tract, 3'-*Plac* and *Ptac* promoters. Theoretically, recombination could occur at any position within identical sequences among P1, P2, and P3 as illustrated in Figure 3B. Recombination between P1 and P2, however, has never been detected even though these two sites share 176-bp identical sequences (uppercase letters underlined in Fig. 3A; open and closed box in Fig.

3B). The only difference between the region P1–P2, which did not show recombination, and the regions P1–P3 and P2–P3, which did show recombination, is that poly(dG)–poly(dC) sequences were not inserted in the region P1–P2, whereas poly(dG)–poly(dC) sequences were inserted in the regions P1–P3 and P2–P3.

Poly(dG)–poly(dC) sequences induce homologous recombination upon transcriptional activation

The effect of the dG tracts on the transcription-dependent recombination was examined in more detail. We studied the frequencies of recombination for plasmid DNAs, pL-GEX and pL-GEX- G_{27} , at varying lengths of time during IPTG treatment in liquid culture of *E. coli* AB2463 (*recA*[−]) cells transfected with one of these DNAs. Bacterial cells (5×10^3) transfected with plasmid DNA were incubated in LB for 1 hr. The cells were then incubated in LB containing 0.5 mM IPTG and ampicillin for 3, 4, 5, 6, 7, 8, or 9 hr and incubated further for 16 hr

without IPTG. The plasmid DNAs were isolated, and the *EcoRV*–*EcoNI* fragments, which were end-labeled at the *EcoNI* site by the Klenow fragment of polymerase I, were separated on a 4% native polyacrylamide gel. To quantify the recombination frequencies in each plasmid, the intensity of the bands was measured by a densitometer, and the recombination frequencies were calculated as described in the legend to Figure 4A. As shown in Figure 4A, a poly(dG)–poly(dC) sequence-inserted construct (pL-GEX- G_{27}) showed 50% recombination after 5 hr [typical data are shown in Figs. 4B (lane 8) and 5A, C (lane 6)] and nearly 100% recombination after 7 hr of exposure to IPTG. On the other hand, no trace of recombination was detected for the control plasmid pL-GEX without poly(dG)–poly(dC) sequence in *recA*[−] cells even after 9-hr IPTG induction in liquid culture. This is in full agreement with our earlier result that no recombination was detected in *recA*[−] cells for the control plasmid isolated from the 16-hr IPTG induction on an agar plate (Fig. 2, lane 5). The growth curve for *recA*[−] AB2463 cells

Figure 4. Recombination frequencies at various time points of IPTG exposure in *recA*[−] cells. (A) Plasmid DNAs, pL-GEX, and pL-GEX- G_{27} , were transfected into *E. coli* AB2463 (*recA*[−]) competent cells. The transfected cells were incubated at 37°C for 1 hr in 3 ml of amp-LB. IPTG was then added to a final concentration of 0.5 mM. After the indicated incubation time with IPTG, 1 μ l of the IPTG-induced cultures was inoculated into 3 ml of fresh amp-LB without IPTG and was incubated for an additional 16 hr. The bacterial growth was monitored at various time points by the OD₅₅₀ measurement. The plasmid DNAs were prepared by the alkaline-lysis method followed by phenol extraction. The *EcoRV*–*EcoNI* fragments of the plasmid DNAs, end-labeled at the *EcoNI* site by Klenow fragment of polymerase I, were separated by a 4% native polyacrylamide gel. The intensities of *EcoRV*–*EcoNI* fragments (1400, 1200, and 400 bp) were quantified by a densitometer from X-ray films. The frequencies of recombination (total recombination frequency: type I plus type II) were calculated as quotient of total intensity of 1200-bp fragments (type I recombination product) and 400-bp fragments (type II recombination product) divided by total intensity of 1400 bp (undeleted *EcoRV*–*EcoNI* fragment), 1200-, and 400-bp fragments. Error bars indicate minimum and maximum values obtained from three experiments. (Δ) pL-GEX; (●) pL-GEX- G_{27} , total recombination frequency. (○) pL-GEX- G_{27} , OD₅₅₀. (B) Southern blot analyses were performed on plasmid DNAs, pL-GEX and pL-GEX- G_{27} , prepared from *E. coli* directly after IPTG induction and after an additional 16-hr incubation without IPTG. First, the *E. coli* cells bearing the plasmid DNAs were cultured overnight. Then 5 ml of the overnight cultures were inoculated into 500 ml of amp-LB and incubated for 2 hr. IPTG was then added to the 500 ml cultures at 0.5 mM and the cultures were incubated for 3–5 hr as indicated. The plasmid DNAs were isolated directly from the 500 ml cultures by the alkaline-SDS lysis method followed by CsCl density gradient centrifugation (lanes 3,6,7). In parallel, 1 μ l of the IPTG-induced cultures was removed after a 5-hr IPTG induction, inoculated into 3 ml of LB without IPTG, and incubated for an additional 16 hr (lanes 2, 4, 8, 9, indicated as +). The plasmid DNAs were then purified by alkaline-SDS lysis followed by CsCl density gradient centrifugation (lane 8) or phenol extraction (lanes 4,9) to examine total DNA not limited to the supercoiled DNA. The plasmid DNAs digested with *EcoNI* and *EcoRV* restriction enzymes were analyzed by the Southern blot method as described in Fig. 2. (Lane 1) λ HindIII DNA size marker; (lane 2–4) pL-GEX; (lanes 5–9) pL-GEX- G_{27} ; (lanes 2) IPTG (−); (lane 5) IPTG, 3 hr; (lane 6) IPTG, 4 hr; (lanes 3,4,7,8,9), IPTG, 5 hr. (b) *EcoRV*–*EcoNI* 3800-bp fragment; (d) *EcoRV*–*EcoNI* 1400-bp fragment; (e) *EcoRV*–*EcoNI* 1200-bp fragment.

Figure 5. Quantitative analysis of recombination products in plasmids containing varying lengths of poly(dG)–poly(dC) sequences. A 5-hr IPTG induction was performed on *E. coli* AB1157 cells bearing plasmids pL-GEX-C/G. The experiments and the calculation for the frequency of recombination are as described in the legend to Fig. 4. The frequencies of type I and type II recombinations were calculated as quotient of intensity of the 1200-bp fragment divided by total intensity of 1400-, 1200-, and 400-bp fragments (type I recombination, hatched bar), and as quotient of 400 bp divided by total amounts (type II recombination, solid bar). For up to 5 hr of induction with IPTG, no trace of recombination was detected in control pL-GEX plasmid in *recA*⁺ cells, and there was no significant difference in recombination frequencies for a dG tract-inserted plasmid between *recA*⁺ cells and *recA*[−] cells (data not shown). (A) pL-GEX, IPTG(−); (lane 2) pL-GEX, IPTG(+); (lanes 3–9) pL-GEX-G_n, IPTG(+); (lane 3) G₁₄; (lane 4) G₂₁; (lane 5) G₂₅; (lane 6) G₂₇; (lane 7) G₃₂; (lane 8) G₃₅; (lane 9) G₄₅. (B) (Lanes 1–8) pL-GEX-C_n, IPTG(+). (lane 1) C₁₄; (lane 2) C₂₁; (lane 3) C₂₅; (lane 4) C₂₇; (lane 5) C₂₈; (lane 6) C₃₂; (lane 7) C₃₅; (lane 8) C₄₅. (C,D) The calculated recombination frequency of pL-GEX-G and pL-GEX-C corresponding to A and B.

indicated that during the first 5-hr IPTG induction, the cell number remained essentially unamplified (Fig. 4A).

There might be selection of cells bearing the deleted constructs over those bearing the original construct during the 16-hr liquid culture without IPTG. During the IPTG induction step, cells containing the plasmid DNA that has the triplex structure may be lethal, and the only cells to survive the induction may be those with a pre-existing deletion. Very few such cells could be amplified during the subsequent 16-hr culture into a dense population. If this assumption is true, then one would expect to find deleted construct to be a very minute constituent of the plasmid DNA purified immediately after the induction step. Thus, the plasmid DNA purified directly after IPTG induction and after an additional growth period should give completely different restriction digestion patterns.

To check the effect of outgrowth, we compared deletion mutation between the pL-GEX-G₂₇ DNA purified from *recA*[−] AB2463 cells immediately after a 5-hr IPTG induction and the same DNA purified from the cells after an additional 16-hr liquid culture without IPTG (outgrowth). We found that the directly purified plasmid DNAs after IPTG induction gave virtually identical results with DNAs purified from the cell culture incubated for an additional 16 hr in terms of the frequency (50%)

and the type of deletion mutations (Fig. 4B, lanes 7,8). We also checked the methods of plasmid purification for the analysis of the recombination events using ethidium bromide–CsCl gradient ultracentrifugation or phenol extraction after alkaline–SDS lysis. These data also showed that the two different plasmid purifications gave the same results (Fig. 4B, lanes 8,9). Deletion mutations for the plasmid DNA purified immediately after 3- and 4-hr IPTG inductions were also examined. After a 3-hr IPTG induction, no deletion was detected. After a 4-hr IPTG induction, however, a significant portion (20%) of the plasmid DNA showed deletion mutation. Again, these results remained unchanged after an additional incubation for 16 hr without IPTG (Fig. 4A,B). This rules out the possibility that cells bearing the deleted constructs are selected during the additional 16-hr liquid culture. It is also unlikely that selection took place during IPTG induction because high frequencies of mutation were already detected during the 4- to 5-hr IPTG-induction period without cell amplification (Fig. 4A). Therefore, rather than selection of cells bearing pre-existing deletions, these results taken together support the notion that poly(dG)–poly(dC) sequences actively participate in the induction of homologous recombination that leads to deletion mutation when the transcription of the downstream gene is activated.

Poly(dG)–poly(dC) sequences induce recombination in a length- and orientation-dependent manner

Poly(dG)–poly(dC) sequences are known to adopt intramolecular dG.dG.dC triplex structures either in purified supercoiled plasmid DNA in vitro or in *E. coli* cells in a length-dependent manner (Kohwi and Kohwi-Shigematsu 1991; Kohwi et al. 1992). This means that under a given superhelical density and ionic strength, poly(dG)–poly(dC) sequences longer than a minimum length adopt a dG.dG.dC triplex, and the longer the dG tracts are, the more readily a triple helix is formed. The triplex formation and the increase in recombination frequency induced by poly(dG)–poly(dC) sequences may be related. To test this idea, we first examined the effect of the lengths of poly(dG)–poly(dC) sequences on recombination. We employed *E. coli* cells carrying constructs containing varying lengths of poly(dG)–poly(dC) sequences and analyzed recombination frequencies in liquid culture after a 5-hr exposure to IPTG. The plasmid pL-GEX- G_n , where $n = 14, 21, 25, 27, 32, 35$, or 45 , was transfected into *E. coli* AB1157-competent cells. The *E. coli* cells were grown, and the plasmid DNAs were purified by phenol extraction and analyzed as described above. The control construct, pL-GEX without dG tracts, did not exhibit any recombination either, with (Fig. 5A, lane 2) or without (Fig. 5A, lane 1) IPTG induction, and only the intact *EcoRV*–*EcoNI* fragment (1400 bp) was detected. On the other hand, for pL-GEX-G plasmids containing dG tracts of 14–45 bp (Fig. 5A), two shorter *EcoRV*–*EcoNI* fragments appeared, which reflect type I and type II recombination upon induction with IPTG (Fig. 5A, lanes 4–9). For up to 5 hr of induction with IPTG, no trace of recombination was detected in control pL-GEX plasmid in *recA*⁺ cells, and there was no significant difference in recombination frequencies for a dG tract-inserted plasmid between *recA*⁺ cells and *recA*[−] cells (data not shown).

The results shown in Figure 5 indicate that type I and type II recombinations exhibit dG tract length dependency. The type I recombination (200 bp deletion) was induced by a short dG tract of 14 bp (pL-GEX- G_{14}) such that 30% of the plasmid population was deleted (Fig. 5A,C, lanes 3). The total recombination frequency increased as the dG length increased, and nearly 100% deletion was obtained with a long dG tract of 45 bp (Fig. 5A,C, lanes 9). The type II recombination (1000-bp deletion) was dramatically enhanced for a dG tract of 21 bp, which resulted in a 50% frequency (Fig. 5A,C, lanes 4). For longer dG tracts, the type II recombination was detected at a low level not exceeding 10% (Fig. 5A,C, lanes 5–9).

The effect of orientation of poly(dG)–poly(dC) sequences on homologous recombination was also examined. A similar experiment was done with the plasmids (pL-GEX-C) containing varying lengths of poly(dG)–poly(dC) in the opposite orientation from the pL-GEX-G construct described above. Although pL-GEX-C showed very high frequency of type I recombination (Fig. 5B,D), there were some differences between pL-GEX-C and pL-

GEX-G. One major difference was found in the length-dependent type II recombination. Although the type II recombination (1000-bp deletion) was detected at very low frequency (<5%) up to 28 bp for pL-GEX-C (Fig. 5B,D, lanes 2–5), a much higher frequency of 50% was detected for pL-GEX- G_{21} (Fig. 5A,C, lanes 4). Such high frequency of type II recombination was never observed for pL-GEX-C at any length of the dC tracts (Fig. 5B,D). Another difference between pL-GEX-C and pL-GEX-G was that the type I recombination occurred at high frequency (30%) even for pL-GEX- G_{14} (Fig. 5A,C, lanes 3), although no recombination was detected for pL-GEX- C_{14} (Fig. 5B,D, lanes 1). Thus, the pL-GEX-G construct is more prone to undergo type I recombination than the pL-GEX-C construct for short poly(dG)–poly(dC) tracts.

These results show that insertion of the poly(dG)–poly(dC) sequences in either orientation results in high levels of recombination frequencies between the two direct repeats separated by either 200 or 1000 bp. The level and the type of recombination, however, depend on both the length and the orientation of the poly(dG)–poly(dC) sequences. Both *recA*⁺ and *recA*[−] cells, after a 5-hr exposure to IPTG, revealed virtually identical results on the dG length-dependent recombination (data not shown).

Recombination is independent of phasing of the dG tracts

In site-specific recombination of λ phage and *E. coli* DNA, it is known that IHF bends DNA, and recombination is sensitive to phasing of the bent DNA (Goodman and Nash 1989; Snyder et al. 1989). In our homologous recombination system, it is also possible that a protein that binds to the dG tract participates in recombination by inducing a bend at the dG tract. If so, phasing of the dG tract relative to the *Ptac* promoter might affect recombination rates. To test this idea, the distance between the 3' end of the dG tracts and +1 site was varied by inserting random-sequence oligomers of varying lengths. The 3' end of the dG tract in pL-GEX- C_{27} is located at −87 from the +1 site of the *Ptac*. The other four plasmid DNAs constructed have 82-, 92-, 108-, and 119-bp distances from the +1 site of the *Ptac* (*P3*). The recombination frequencies were analyzed as described in the legend to Figure 4A. As shown in Figure 6, the plasmid DNAs with a half helical turn difference (−5 bp, lane 1; +5 bp, lane 3) from the original pL-GEX- C_{27} (lane 2) showed the same recombination frequency. Moreover, the plasmid DNAs with the same phasing as pL-GEX- C_{27} but longer spaces (+10 bp, lane 4; +21 bp, lane 5; +32 bp, lane 6) also showed virtually the same frequency. Therefore, the homologous recombination mediated by the dG tracts does not display the DNA turn dependence seen in site-specific recombination (Goodman and Nash 1989) or in the transcriptional activation of the SV40 DNA early promoter in a eukaryotic system (Takahashi et al. 1986). Also, in the absence of poly(dG)–poly(dC) sequences, none of the random sequences em-

Figure 6. Phasing effect of the dG tracts relative to the PtaC on recombination. Varying lengths of random sequences were introduced between PtaC promoter and dG tracts in pL-GEX-C₂₇. Those DNAs were subjected to the recombination analysis with IPTG induction, and total recombination frequencies were calculated as described in the legend to Fig. 4. The distance of the space was counted from the +1 site of the PtaC promoter to the 3' end of the poly(dG)–poly(dC) sequence. Error bars indicate minimum and maximum values obtained from three experiments. (Lane 1) 82 bp; (lane 2) 87 bp (original pL-GEX-C₂₇); (lane 3) 92 bp; (lane 4) 97 bp; (lane 5) 108 bp; (lane 6) 119 bp.

ployed in this experiment to alter the distance stimulated recombination (data not shown).

Transcription-induced dG.dG.dC triplex structure is detected in *E. coli* cells

Insertion of poly(dG)–poly(dC) tracts between homologous sequences induces recombination of these sequences after transcriptional activation of a downstream gene in *recA*[–] cells. We infer from these results that negative superhelical strain accumulated by active transcription of a gene stabilizes the formation of an intramolecular dG.dG.dC triplex structure by poly(dG)–poly(dC) sequences, which consequently brings remote sequences together to stimulate recombination. If this is the case, we should be able to detect the dG.dG.dC triplex formation in *E. coli* cells in a transcription-dependent manner. To detect the triplex in *E. coli* cells, we employed chloroacetaldehyde (CAA), an analog of bromoacetaldehyde (BAA), which specifically detects unpaired adenine and cytosine bases. These chemicals have been used for detecting non-B DNA structures including intramolecular triple helices both in vitro and in vivo (for review, see Kohwi-Shigematsu and Kohwi 1992). When poly(dG)–poly(dC) sequences form a dG.dG.dC triplex, cytosines over the 5' half of the dC tract that are not involved in the triplex formation are unpaired and are reactive with CAA. Thus, dG.dG.dC triplex formation can be demonstrated by the cleavages of DNA at

these CAA-reacted cytosine residues, as shown by the appearance of new bands within sequencing ladders. An intramolecular dG.dG.dC triplex, which forms only under negative superhelical strain, was detected previously in *E. coli* cells after chloramphenicol treatment when maximal unconstrained superhelical density was achieved (Kohwi et al. 1992).

We anticipated that the recombination might proceed rapidly once a dG.dG.dC triplex forms and that these two events might be coupled. Therefore, to detect a transcription-dependent dG.dG.dC triplex formation in *E. coli* cells at any given time point, we employed P1- and P2-deleted plasmid DNAs that were derived from pL-GEX-C DNAs (pΔ-GEX-C₁₄, -C₂₁, -C₂₇, -C₃₂, and -C₃₅) so that recombination could not take place even in a 5-hr IPTG induction. As shown in Figure 7, no CAA reacted sites were detected in dC tracts in the negative control (Fig. 7, lane 8; pΔ-GEX-C₃₅, without IPTG induction or chloramphenicol treatment, but with CAA treatment). On the other hand, the plasmid pΔ-GEX-C₃₅ DNA in *E. coli* cells treated with chloramphenicol (Fig. 7, lane 7) showed a typical CAA reaction pattern, indicating a dG.dG.dC triplex formation, such that cytosine residues

Figure 7. Detection of a dG.dG.dC triplex structure in *E. coli* cells. Plasmid DNAs modified with CAA in situ were prepared as described in Materials and methods. The HindIII–EcoRV fragments, 3'-end radiolabeled at the HindIII site (dC tracts were labeled), were treated with formic acid and subsequently cleaved by piperidine (Maxam and Gilbert 1980). CAA-treated dC residues over the 5' half of the dC tract were detected as new cleavage sites in the purine ladder. This pattern of CAA reactivity indicates the intramolecular dG.dG.dC triplex formation (Kohwi and Kohwi-Shigematsu 1988; Kohwi et al. 1992). (Lanes 1–5) IPTG, 3 hr; (lane 6) IPTG, 5 hr; (lane 7) chloramphenicol, 2 hr; (lane 8) noninduced control. (Lane 1) pΔ-GEX-C₁₄; (lane 2) pΔ-GEX-C₂₁; (lane 3) pΔ-GEX-C₂₇; (lane 4) pΔ-GEX-C₃₂; (lanes 5–8) pΔ-GEX-C₃₅.

over the 5' half of poly(dC) sequence were CAA modified and cleaved by the formic acid/piperidine reaction (Kohwi and Kohwi-Shigematsu 1988; Kohwi et al. 1992). When the same plasmid DNA was treated with IPTG for 3 hr in vivo, it showed the same CAA reaction pattern (Fig. 7, lane 5) as seen in the chloramphenicol-treated positive control (Fig. 7, lane 7). This CAA reaction pattern for dG.dG.dC triplex formation in *E. coli* cells was also observed in a 3-hr IPTG induction in 32-bp length (Fig. 7, lane 4) but not in 27-bp length (Fig. 7, lane 3), 21-bp length (Fig. 7, lane 2) or 14-bp length (Fig. 7, lane 1) DNA fragments. A 5-hr IPTG induction, however, reduced CAA reactivity (Fig. 7, lane 6). Similar prolonged induction with chloramphenicol also failed to detect a dG.dG.dC triplex, suggesting that superhelical environment suitable for the triplex formation is transient (data not shown). The data indicate that the transcriptional activation induces dG.dG.dC triplex formation in vivo. This demonstrates that negative superhelical strain is created as a result of transcriptional activation of the downstream gene. The data also suggest that the population of *E. coli* having the triplex structure in vivo may be small at any given time, and detection of the triplex for shorter dG tracts may be difficult. The triplex formation is an earlier event than recombination, which shows a sharp rise after a 4-hr IPTG induction and achieves much higher frequency after 5 hr (Fig. 4A). Thus, these two events do not seem to be temporally coupled. The delay in the recombination event may be caused by the time required for the actual recombination to take place after a full assembly of the recombinational machinery.

Discussion

Transcription results in the triplex formation and homologous recombination in a RecA-independent manner

We employed poly(dG)–poly(dC) sequences that form an intramolecular triplex structure in response to negative supercoiling to study whether a non-B DNA structure formation mediates homologous recombination stimulated by transcription. We have shown that by inserting poly(dG)–poly(dC) sequences between direct repeats in the 5' region of the promoter, the transcriptional activation of a bacterial gene induces homologous recombination of its upstream sequences in a RecA-independent manner. In the absence of poly(dG)–poly(dC) sequences, no recombination was induced by transcription in *recA*[−] cells.

Direct chemical probing showed that an intramolecular dG.dG.dC triplex forms in vivo after transcriptional activation (Fig. 7). This structure was detected previously in *E. coli* cells only under high unconstrained negative superhelical density achieved after chloramphenicol treatment (Kohwi et al. 1992). This shows that a local negative superhelical strain is created by transcriptional activation of a gene and stabilizes a triple helix in the 5' region, in agreement with the twin-supercoiling model

proposed by Liu and Wang (1987). The intramolecular dG.dG.dC triplex formation results in a sharp folding of the DNA. Therefore, this structure could bring remote sequences into close proximity to induce recombination (models shown in Fig. 8).

To examine possible involvement of RecA protein in our recombination system, we studied recombination events using several *recA*[−] *E. coli* strains, in addition to AB2463 (with the same genetic background as AB1157 *recA*⁺), HB101, and DH5 α . In *recA*[−] cells, transcription-induced recombination was observed only in the poly(dG)–poly(dC) sequence-inserted construct, pL-GEX-C/G, and not in the control pL-GEX plasmid. It is conceivable that when two homologous sequences are aligned by sharp folding of DNA as a result of intramolecular triplex formation, RecA may no longer be needed to initiate DNA pairing. There are other studies describing recombinations that occur in *recA*[−] cells (for review, see Clark and Low 1988; Petes and Hills 1988). Such recombination involves short regions of homologies and is thought to proceed by another pathway such as slippage of pairing during DNA replication, or illegitimate recombination events catalyzed by gyrase (Albertini et al. 1982; Marvo et al. 1983; Chiba et al. 1989). The RecA independent homologous recombination that we have studied appears to be mediated by the non-B DNA structure formation.

Model for homologous recombination mediated by transcription-induced triplex

The model for the role of the triplex formation in recombination is presented in Figure 8 (a double-stranded form of poly(dG)–poly(dC) sequence is shown by an open triangle, and a triple-helix form is shown by a solid triangle). When the Ptac promoter (P3) is activated by IPTG induction, the negative superhelical strain accumulates at the 5' region of the promoter [Fig. 8 (2); Liu and Wang 1987] and induces a poly(dG)–poly(dC) sequence to form a triple-helix structure [Fig. 8 (2)]. The intramolecular triplex forms via sharp folding of DNA into halves. This would bring distant sequences into close proximity enabling them to align so that either type I or type II recombination is promoted [Fig. 8 (3), I or II, respectively]. The stability of the dG.dG.dC triplex increases as the length of the dG tract increases. Therefore, the length dependency of the dG tracts on recombination frequency supports the idea that the triplex formation induces recombination [Figs. 5 and 8 (3)].

We have also shown orientation dependency for the effect of poly(dG)–poly(dC) sequences on homologous recombination. A significant level of type I recombination was detected even for the pL-GEX-G₁₄ containing a short dG tract of 14 bp, but there was no recombination in the comparable construct containing a dC tract of 14 bp (Fig. 5A,C, lanes 3 for -G₁₄; Fig. 5B,D, lanes 1 for -C₁₄). The dG₂₁-containing construct, pL-GEX-G₂₁, revealed a substantially high level of type II recombination not found in pL-GEX-C₂₁ (Fig. 5A,C, lanes 4 for -G₂₁; Fig. 5B,D,

Figure 8. Model for type I and type II recombination. Plasmid DNAs of pL-GEX-C/G are represented by horizontal rules (1). P1, P2, and P3 represent the homologous sequences of 5'-*lac*, 3'-*lac*, and *tac* promoters, respectively, as indicated by open boxes in Fig. 3A. (Δ) Double-stranded poly(dG)-poly(dC) sequences. Once the Ptac promoter is activated by IPTG, two supercoiled domains of opposite signs are generated: A positively supercoiled domain is formed in front of the transcribing RNA polymerase [at the 3' regions of P3 (+)], and a negatively supercoiled domain is formed behind [at the 5' regions, (-)] (2) twin-domain model by Liu and Wang 1987]. When negative torsional strain accumulates in the region containing poly(dG)-poly(dC) sequence, a dG.dG.dC triplex is formed [2 and 3, (▲)]. The triplex formation brings P2 and P3 that are 200 bp apart into close vicinity to stimulate recombination [type I recombination, 3(I)]. The triplex formation also brings P1 and P3 separated by 1000 bp close together to stimulate recombination [type II recombination 3(II)]. For the pL-GEX-G construct, where dG tracts are on the coding strand with respect to the Ptac promoter (P3), the dG.dG.dC triplex formation affects unpairing on P3 region unidirectionally (4A). Depending on the extent of the unpairing induced by the dG tracts, one could have stimulation or attenuation of transcription. For example, an optimal dG length (dG_{21}) may induce base-unpairing just upstream of the Ptac promoter, which might help RNA polymerase to initiate transcription. This would result in more active transcription and lead to more superhelical strain 5' of the gene to stabilize the triplex. Any longer dG tracts may cause more extensive unpairing within the Ptac promoter and this might result in partial inactivation of the Ptac promoter. For the pL-GEX-C construct, where dC tracts are on the coding strand, dG.dG.dC triplex formation creates unpairing at opposite side of the dC tract from P3 (4B). The activity of the Ptac promoter may not be affected by unpairing of the triplex formation. Therefore, in this case, the frequency of recombination is more a direct reflection of the propensity of the triplex formation, which is in turn dependent on the dG tract length. (●) Hydrogen bonds in the triplex and in the neighboring sequences.

lanes 2 for $-C_{21}$). How could this be explained? The inherent nature of poly(dG)-poly(dC) sequences that induce DNA unpairing in a unidirectional manner may play an important role. Once the dG.dG.dC triplex forms, the remaining 5' half of cytosine residues that do not form the triplex are exposed and unpaired. This induces unpairing in the neighboring region 5' of the poly(dC) residues. The 5' half of cytosine strand and the complementary 3' half of the guanine strand that forms a triplex must change their mutual orientation in space so that they can be base-paired again in the 3' neighboring region of the dG tracts [Fig. 8 (4); Kohwi and Kohwi-Shigematsu 1988]. The extent of unpairing depends on the dG tract length: The longer the dG tract is, the more neighboring sequences become unpaired (Kohwi-Shigematsu and Kohwi 1985). This unidirectional unpairing is illustrated in Figure 8 (4A) for pL-GEX-G and in Fig. 8 (4B) for pL-GEX-C. It is possible that the pL-GEX-G construct with an optimal dG length (dG_{21}) induces base-unpairing or unwinding just upstream of the Ptac promoter. This may stimulate transcription because base-unpairing at an appropriate position might help RNA

polymerase to initiate transcription. It has been demonstrated that DNA relaxation in the -10 region in a *gyr* gene promoter is responsible for the stimulation of transcription (Menzel and Gellert 1987). For the pL-GEX- G_{21} construct, an increase in transcription rate may be achieved by this mechanism. If so, this would increase the superhelical strain 5' of the gene, which in turn stabilizes the triplex, more, and result in a higher frequency of recombination. This would not be possible for pL-GEX- C_{21} , which promotes base-unpairing distal from the promoter. As the length of the dG tracts increases in pL-GEX-G constructs, the structural effect would extend more into the promoter region and could result in transcriptional attenuation. This effect may be counterbalanced by the increase in stability of the triplex as the dG length increases. The orientation dependency of the poly(dG)-poly(dC) sequences on recombination could also be explained by the possibility that a triplex in the pL-GEX-G construct in which the unpaired region is proximal to the promoter may be easier to form. This may explain why pL-GEX- G_{14} show recombination but not pL-GEX- C_{14} .

Biological consequences of transient supercoiling generated by transcription

Although DNA supercoiling of opposite signs generated by the process of transcription is transient in nature, this could exert a variety of effects on biological processes and functions involving DNA. Clark and Felsenfeld (1991, 1992) proposed a model showing that in the case of mammalian cells, the histone octamer is transferred from the positively supercoiled DNA in the path of the transcribing polymerase to the negatively supercoiled DNA behind it. This was based on the observation that nucleosome cores are much more stable on negatively supercoiled DNA compared with positively supercoiled DNA. The twin-supercoiled-domain model also predicts that promoters in the same topological domain may influence one another via local effects on DNA supercoiling. In *E. coli*, such a notion of one promoter controlling the activity of a second promoter provides an explanation for the anomalous regulation of the *leu-500* promoter (Lilley and Higgins 1991).

Non-B DNA structures, such as left-handed Z-DNA and cruciform structures, are known to form in *E. coli* cells or in vitro as a consequence of supercoiling generated by transcription (Droge and Nordheim 1991; Dayn et al. 1992; Rahmouni and Wells 1992). Previously, we reported results suggesting that the DNA structure altered by local supercoiling can affect the level of transcription in mammalian cells (Kohwi and Kohwi-Shigematsu 1991). When placed 5' of a reporter gene, the triplex-forming poly(dG)–poly(dC) sequences augmented the level of transcription in mammalian cells in a length-dependent manner. In vivo competition assay suggests that a *trans*-acting factor binds a double-stranded poly(dG)–poly(dC) sequence but not when it is forming a triplex. The observation that a dG tract of 25- to 30-bp enhanced transcription, but one of >32 bp abruptly abolished this enhancement, strongly suggested that an altered DNA structure could affect the level of transcription.

In this study, we have not only demonstrated transcription-induced intramolecular dG.dG.dC triplex formation in *E. coli* cells but also transcription-induced homologous recombination of upstream sequences mediated by the presence of poly(dG)–poly(dC) sequences. This model experiment may provide an important biological implication: Sequences that readily form non-B DNA structures under superhelical strain can be responsible for stimulating homologous recombination of the flanking sequences in response to transcription of nearby genes. It is important to mention that even in a wild-type DNA topoisomerase background, triple-helix structure formation and subsequent recombination events are established by transient local supercoiling generated by transcriptional activation before topoisomerases relax the DNA. Not all non-B DNA structures would stimulate recombination. Depending on the exact nature of the structure, the effect would be different. For example, in some cases, it could keep homologous sequences apart to inhibit recombination. A fused or double triple helix

(Kohwi-Shigematsu and Kohwi 1991; later called a nodule structure by Panyutin and Wells 1992), if formed in vivo, would keep 5' and 3' neighboring sequences apart. Because there are many stretches of sequences that form different types of non-B DNA structures, it is likely that transcription-stimulated recombination is mediated by some of those sequences in vivo.

Materials and methods*Plasmids*

Plasmid DNA, pGEX-2T was purchased from Pharmacia. Plasmid DNA, Bluescript II KS+ was purchased from Stratagene, and the *EcoRV*–*PvuII* fragment containing the *lac* promoter was subcloned into *SspI* site of the pGEX-2T, which was partially digested. The new construct, which contained the pGEX-2T *Ptac* promoter and the Bluescript *lac* promoter in the same direction, was selected and named as pL-GEX. Various lengths of poly(dG)–poly(dC) sequences were introduced into the plasmid pL-GEX. *EcoRI*–*BamHI* fragments from pCAT(C/G)_n (Kohwi and Kohwi-Shigematsu, 1988) were blunt-ended by the Klenow large fragment and inserted at a blunt-ended *XhoI* site of pL-GEX. Plasmids pL-GEX-G and pL-GEX-C indicate either the dG tract on the top strand or the dC tract on the top strand with respect to the *Ptac* promoter, respectively. To change the length between dG tracts and *Ptac* promoter, *SalI* and *HindIII* fragments generated by synthetic oligomers [TCGACGGATAAGCT (Fig. 6, lane 1); TCGACGGATCAGACTCGATAAGCT (Fig. 6, lane 3); TCGACGGATCAGACATGACTCGATAAGCT (Fig. 6, lane 4); TCGACGGATCAGACATGACCGCTTGACATGTCGATAAGCT (Fig. 6, lane 5); TCGACGGATCAGTACTCACGCAGACATGACCGCTTGACATGTCGATAAGCT (Fig. 6, lane 6)] were inserted in the *SalI*–*HindIII* sites of pL-GEX-C₂₇.

Deletion mutation assay of recombination events

Plasmid DNAs, pL-GEX, pL-GEX-C, and pL-GEX-G, were transfected into *E. coli* AB1157 (*recA*[−]) or AB2463 cells (*recA*[−], with the same genetic background as AB1157 *recA*⁺, Howard-Flanders and Theriot 1966; Moriya et al. 1988). Both cells were kindly provided by Drs. M. Moriya and A.P. Grollman. The transfected cells were incubated at 37°C for 1 hr in 3 ml of amp–LB, and IPTG was then added to the cultures at a 0.5 mM of final concentration. After incubation for the time indicated in the figures, typically 5 hr, 1 μl of the IPTG-induced cultures was inoculated to 3 ml of fresh amp–LB and incubated at 37°C for 16 hr without IPTG. The plasmid DNAs were prepared from the overnight cultures by the alkaline–SDS method (Maniatis et al. 1982). The purified plasmid DNAs were labeled at the *EcoNI* site by the Klenow large fragment and [α -³²P]dATP followed by digestion with the *EcoRV*. The *EcoNI*–*EcoRV* fragments were separated by 4% native polyacrylamide gel electrophoresis. The intensities of the radioactivity of the fragments were quantified by a densitometer (LKB UltrosanXL) from X-ray films. For sequence analysis, the *EcoNI*–*EcoRV* fragments were isolated from the native polyacrylamide gel and subjected to the Maxam–Gilbert chemical reaction (Maxam and Gilbert 1980) by use of formic acid/piperidine (for dG and dA detection).

Southern blot analysis

Plasmid DNAs were digested with *EcoNI* and *EcoRV*. The digested DNAs were analyzed by the method of Southern (1975). The DNAs were separated by 1% agarose gel and blotted onto

the nylon membrane (Zeta-probe, Bio-Rad) by alkaline transfer. The blotted membrane was UV cross-linked by Stratalinker-2400 and hybridized in 50% formamide in a roller bottle (Hoefer Scientific Instruments) at 42°C with ³²P-labeled plasmid DNA, pL-GEX, and washed with 0.2× SSC (20× SSC: 175.3 grams of sodium chloride, 88.2 grams of sodium citrate per liter at pH 7.0) and 0.1% SDS at 68°C for 1 hr.

Analysis of triplex structure in *E. coli* cells

P1–P2 sequence-deleted plasmid DNAs were constructed from pL-GEX-C_n plasmid DNAs. Briefly, the P2 sequence was removed first by *Kpn*I and *Bsp*MI digestion and religation, and then the P1 sequence was removed by the method of oligonucleotide mediated PCR with *Nar*I and *Bsu*36 sites. The new deletion plasmid DNAs, pΔ-GEX-C₁₄, pΔ-GEX-C₂₁, pΔ-GEX-C₂₇, pΔ-GEX-C₃₂, and pΔ-GEX-C₃₅, no longer have extensive homologous sequences, but poly(dG)–poly(dC) and P3 sequences remain intact (~550 bp shorter than the original). To detect dG.dG.dC triple-helix structure *in vivo*, we employed the method of Kohwi et al. (1992). Briefly, AB1157 *E. coli* cells carrying each pΔ-GEX-C_n plasmid DNA were grown for 2 hr in M9 media supplemented with casamino acids. The transcription was then induced with 0.5 mM of IPTG for 3 or 5 hr. *E. coli* cells were washed with phosphate-buffered saline (PBS) with 2 mM MgCl₂ and 0.2 mM IPTG at room temperature. Immediately after the wash, the *E. coli* cells were treated with a chemical probe specific for unpaired DNA bases, CAA, at 37°C for 30 min. The plasmid DNAs were prepared by the alkaline–SDS lysis method (Maniatis et al. 1982) followed by 2× CsCl density gradient centrifugation. CAA-reacted sites in the plasmid DNAs were analyzed by the method of Kohwi and Kohwi-Shigematsu (1988).

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