The Effects of Nucleotide Sequence Changes on DNA Secondary Structure Formation in Escherichia coli Are Consistent With Cruciform Extrusion in Vivo

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Manuscript received October 6, 1993
Accepted for publication February 10, 1994

ABSTRACT

The construction in bacteriophage λ of a set of long DNA palindromes with paired changes in the central sequence is described. Identical palindrome centers were previously used by others to test the S-type model for cruciform extrusion in vitro. Long DNA palindromes prevent the propagation of carrier phage λ on a wild-type host, and the sbcC mutation is sufficient to almost fully alleviate this inviability. The plaque areas produced by the palindrome containing phages were compared on an Escherichia coli sbcC lawn. Central sequence changes had a greater effect upon the plaque area than peripheral changes, implying that the residual palindrome-mediated inviability in E. coli sbcC is center-dependent and could be due to the formation of a cruciform structure. The results argue strongly that intrastrand pairing within palindromes is critical in determining their effects in vivo. For the same data suggests that DNA loops in vivo may sometimes contain two bases only.

DNA palindromes longer than 100-150 base pairs (bp) cannot be propagated in wild-type Escherichia coli. Either a replicon with a long DNA palindrome is so poorly replicating that it is inviable, or the palindrome itself is so unstable that it undergoes partial or complete deletion (Collins 1981; Lilley 1981; Leach and Stahl 1983; Leach and Lindsey 1986; Shurvin et al. 1987; Lindsey and Leach 1989). These phenomena are termed inviability and instability respectively, and their relationship is poorly understood.

The biological effect of palindromic sequences is alleviated in certain host strains. λ phage carrying a long palindrome, although unable to plate on wild-type E. coli, were found to be viable on a recBC sbcB host (Leach and Stahl 1983). Subsequently this strain was found to have an unrecognized mutation in the sbcC gene (Lloyd and Buckman 1985). Further work revealed that the sbcC mutation by itself is necessary and sufficient to allow plating (Chalker et al. 1988). SbcC is now known to be one partner in a two gene system with sbcD. Together they have been implicated in palindrome-mediated inviability, in addition to their role in the cosuppression of recombination deficiency in recBC mutants (Lloyd and Buckman 1985; Gibson et al. 1992).

DNA palindromes have the potential to adopt cruciform structures, and there is no doubt that cruciform structures do exist in vitro (Gellert et al. 1979; Lilley 1980). For palindromes of average base composition under moderate salt concentrations the S-type pathway for cruciform extrusion in vitro has been proposed, and it is illustrated in Figure 1. Changes to the central sequence of a palindrome affect the in vitro extrusion kinetics to a greater degree than more peripheral changes (Murche and Lilley 1987; Courey and Wang 1988; Zheng and Sinden 1988). A plausible explanation is that a central AT to GC change raises the free energy of the transition state, whereas outside the center a similar change has little effect upon transition state formation because the altered DNA usually remains unmelting during that step (Murche and Lilley 1987). Whether cruciforms exist in vivo has been in dispute until recently. Initial reports failed to find any evidence for such structures (Courey and Wang 1983; Sinden et al. 1983), but in retrospect this may have been due to the insufficient sensitivity of the assays used. In addition, the palindromes studied were short (50-60 bp) or of a low AT content so that the cruciform might not be expected to be the predominant structure. Generally, unusual conditions and AT-rich sequences have been required to detect cruciform structures for short (<100 bp) palindromes (Panayotatos and Fontaine 1987; McClellan et al. 1990; Dayn et al. 1991; Zheng et al. 1991; Sinden et al. 1991). However, evidence consistent with the formation of cruciform structures under normal conditions by long palindromes of an average base composition has recently been obtained (Chalker et al. 1993). These results gave preliminary indications that the pathway by which palindromes may cause inviability is center-dependent.

We have now further tested the hypothesis that the residual palindrome-mediated inviability in an sbcC host is center-dependent, by measuring the effect of central sequence changes in a palindrome on the relative plaque size of the carrier λ. These results argue that intrastrand pairing between the arms of a palindrome is critical in determining its effect on inviability, and are consistent with cruciform extrusion in vivo. The same data suggest that the structure of a DNA loop in vivo is sequence dependent, and in some cases the loop may consist of two residues only.
vectors:

MATERIALS AND METHODS

Media, bacterial strains, bacteriophage strains and plasmid vectors: Casitone-agar (CA) medium contained 10 g Bacto-casitone, 10 g Bacto-agar (both from Difco Laboratories), and 14.7 g NaCl (250 mM final concentration) per liter. The added salt was found to accentuate the differences between bacteriophage strains. All plates were poured at 46°C, and to a volume of exactly 50 ml. A plating took place 3 days after pouring, and before use each stack of plates was randomly reordered. These precautions were necessary because small variations between plates can markedly affect the size of plaques on a lawn. CA top medium contained half the quantity of Bacto-agar (5 g/liter).

The bacterial strain used for the plaque size assay was N2264 (AB1157 sbcC201 phoR::Tn10), and was obtained from R. G. Ll oid. The palindromic containing phages were all derived from DRL133 λ pal sbcC b857 (Chalker et al. 1993). DRL133 was crossed with DRL152 λ sbcC b857 χ C153 to introduce a Chi site. The resultant phage DRL167 λ sbcC b857 χ C153 produced considerably larger plaques on E. coli sbcC than DRL133 due to the effect of Chi on production of packageable DNA. They were sufficiently large to enable the area of individual plaques to be measured with an image analyzer. The palindromic in DRL167 is a 462-bp perfect inverted repeat, flanked by two EcoRI sites and with a unique central SacI site. The SacI site of DRL167 was used to insert oligonucleotide sequences obtained from the OsWel DNA Service (T. Brown, Edinburgh). To aid the identification of new clones, the inserts were designed so that the cloning procedure disrupted the SacI site, and for positive identification another restriction site was introduced into the center. The oligonucleotides were not phosphorylated so as to avoid insertion of multimers. The central sequences of the palindromic phage are described in Figure 2A and were based on the bke series that Murchie and Lilley (1987) constructed to test the S-type extrusion pathway. The phage were named according to the corresponding bke parents.

The center of each palindrome was subcloned and sequenced to confirm that it was as predicted. The palindromic was gel purified after restriction with EcoRI (or Avai and Pael for the palindromes in an EcoRI center). A 92-bp TaqI fragment containing the palindrome center was then ligated into the Acl site of pMS2B (a derivative of pUC18; D. R. F. Leach, M. Shaw and C. Blake). It is illustrated in Figure 2B. The plasmids with inserts corresponding to the center of the palindrome were identified using DNA minipreps and restriction analysis. The sequences of all the plasmids with inserts were as predicted, with no multiple inserts being detected. This series of plasmids was named to match the AAD phage (e.g., pADbke and pAD16T). All manipulations used standard methods, as detailed in Sambrook et al. (1989).

Bacteriophage plating: An overnight culture of E. coli sbcC was diluted 1:10 in L-broth supplemented with 5 mM MgSO₄/0.2% maltose and grown for 140 min at 37°C. An equal volume of 10 mM Tris-HCl (pH 8), 10 mM MgSO₄ was added. The diluted cultures were stored at 4°C and used within 3 days. Aliquots of 0.25 ml of the cell suspension were incubated with a suitable dilution of phage at 37°C for 20 min, and then poured onto CA plates in 2 ml of molten CA top medium. These conditions were chosen to maximize preadsorption of bacteriophage. After an overnight incubation at 37°C, the area of individual plaques on the cell lawn was measured using a Quantimet 970 Image Analyser (C. Jeffrey, Edinburgh). Approximately 60–100 plaques per plate were analyzed (12 plates for each bacteriophage strain).

Cruciform extrusion kinetics: Kinetic studies were performed according to Murchie and Lilley (1987) with some variations, the major difference being that the SI nuclease was...
replaced by T4 exo nuclease VII (T4 endoVII; a gift from B. Kemper) which cleaved at the four-way junction of the DNA cruciform under the conditions used. Supercoiled, cruciform-free plasmid DNA was isolated from an overnight 0.5-liter culture and then purified through two rounds of CsCl-ethidium bromide isopycnic centrifugation. The ethidium bromide was removed with seven or more rounds of butanol-1-01 extraction on ice, followed by extensive dialysis in 10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA at 6°C. The plasmid DNA was stored at -70°C and thawed slowly at 6°C prior to any experiments.

Cruciform extrusion experiments were initially carried out in a 10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA buffer containing a known concentration of NaCl. To determine the extrusion salt optimum the plasmid DNA was incubated in the buffer for a given time period with NaCl added to a concentration of between 35 mM and 75 mM. Subsequently, in vitro cruciform extrusion experiments were performed in a more physiological buffer (buffer P; 150 mM potassium glutamate, 4 mM magnesium acetate, 10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA). After incubation, the sample was rapidly transferred to ice to terminate the reaction. The reaction constant for each cruciform was ascertained by incubating the plasmid DNA in the appropriate buffer, and then withdrawing aliquots onto ice over a given time period. All extrusions took place at 37°C (±0.01) in a Grant LTD6 waterbath. The samples were then diluted into T4 endoVII reaction buffer (0.5 mM Tris-HCl pH 8.0, 50 mM NaCl, 10 mM MgCl2, 1 mM dithiothreitol, 5 mM spermidine, 250 µg/ml bovine serum albumin; Picklesley et al. 1990) and incubated with 4 units T4 endoVII at 16°C for 40 min. No cruciform extrusion was detectable in this buffer at 16°C. After this, the DNA samples were ethanol precipitated, resuspended in a suitable restriction buffer, and digested to completion with ScaI. The DNA samples were loaded onto a 1.5% agarose gel vertical electrophoresis unit and run for approximately 2 hr at 70 V. The gel was stained in 1 µg/ml ethidium bromide and photographed on a UV transilluminator with Ilford HP5 film. The proportion of fragments arising from the T4 endoVII-specific cleavage was determined by assaying the relative intensity of bands on the gel. Primer annealing took place at 70°C in a dry ice bath. Primer annealing took place at room temperature during the labeling step and the extension reactions were run at 45°C to minimize secondary structure formation.

RESULTS

Construction of a set of palindromes with paired changes in the central sequence: A series of long perfect palindromes, differing only in their central sequence, was constructed in λ phage to test the prediction that palindrome mediated inviability is center-dependent. The palindrome centers of the λ phage were related to the bke series of palindromes in pAT153 that had previously been used to test the S-type model for cruciform extrusion in vitro (Murche and Lilley 1987). As shown in Figure 2A, the central 12 bp of the palindromes constructed in λ were identical to the equivalent bke centers in pAT153, but the adjoining palindromic arms of the λ constructs were different and over 10 times longer.

Plating behavior of palindrome phage: The phenotype conferred upon the λ by the palindrome was assessed by plating on a lawn of N2864 sbcC201, where differences in plaque size between the various λ phage were visible. These palindromic phage were inviable on wild-type hosts. Plaque area (under the standardized conditions used here) is assumed to be an indirect product of the λ burst size, and thus a measure of the viability of the palindromic phage. The plaque area was initially assessed on CA plates with the standard NaCl concentration (5 g/liter). A higher salt concentration (14.7 g/liter) was subsequently used for all the plaque assays reported here because it was found to amplify the differences. The areas of individual plaques were assessed accurately for each strain using an image analyzer. A graph of cumulative frequency plotted against plaque area is presented in Figure 3 for four of the strains. The median was the most reproducible parameter since it minimizes the effects of outliers. Outliers were relatively common in this analysis for two reasons: λ phage that are initially unadsorbed produce pinprick plaques on the cell lawn, while revertants (phage that have lost the palindrome) produce very large plaques. The plaque assay median determined for each strain is presented in Table 1 and schematically illustrated in Figure 4A.

Central base-pair changes had the greatest effect on the plaque size. Figure 4A clearly demonstrates this center dependence: λAD10G had larger plaques than

![Figure 3](image-url)
AAD13G, but smaller plaques than AADbke. Significantly, this pattern also included AAD15C and AAD15C16C. Clearly, however, the precise determinants of the degree of inviability of a palindromic-containing λ phage are different from those that govern the rate of cruciform extrusion in vitro and seem inconsistent with a simple S-type mechanism. The central sequence of the palindrome had the predicted effect on plaque size only for changes to the very central 2 bp. Those plaques than AADbke (central sequence AGAATTCT) a palindrome may be critical in determining the level of a 5'-AT-3' or a 5'-TA-3' at the very center of the palindromes:

A cruciform extrusion in vitro of two related series of palindromes: It was necessary to confirm that the central changes in λ had the same effect on in vitro extrusion as the bke centers in pAT153. Since long DNA palindromes are unstable in plasmid vectors (even with the sbcC mutation), it was not possible to use the full length palindromes. Instead a 32-bp TaqI fragment containing the A palindrome center was subcloned into the AccI site of pMS2B (forming the PAD series of plasmids), then sequenced to check that the constructs were as predicted. The extrusion kinetics of the palindrome centers in the PAD plasmids were compared with the similar sequences to pAT153, (NaCl buffer) PAD series (NaCl buffer) PAD series (buffer P) Median plaque area/mm²

<table>
<thead>
<tr>
<th>Mutant</th>
<th>k (error)</th>
<th>Exp</th>
<th>TN/2 (min)</th>
<th>NaCl optimum (M)</th>
<th>k (error)</th>
<th>Exp</th>
<th>TN/2 (min)</th>
<th>NaCl optimum (M)</th>
<th>k (error)</th>
<th>Exp</th>
<th>TN/2 (min)</th>
<th>NaCl optimum (M)</th>
<th>Median plaque area/mm²</th>
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<tr>
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<tr>
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<td>-5</td>
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<tr>
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<td>114</td>
<td>75</td>
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<td>-4</td>
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<tr>
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<td>8</td>
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<td>-4.8 (0.53)</td>
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<td>-3</td>
<td>9.6</td>
<td>0.41</td>
<td></td>
</tr>
<tr>
<td>10G</td>
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<td>-4</td>
<td>81</td>
<td>50</td>
<td>-2.4 (0.26)</td>
<td>-4</td>
<td>48.1</td>
<td>45</td>
<td>ND</td>
<td>ND</td>
<td>0.33</td>
<td>0.33</td>
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</table>

Rate constants are expressed as k(±standard error) X 10⁶. Half-times were calculated from ln 2/k. ND, k not determined.

TABLE 1
Kinetics of cruciform extrusion and plaque assay data for the palindrome plasmids and phage

Cruciform extrusion

<table>
<thead>
<tr>
<th>Mutant</th>
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<th>Exp</th>
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<th>NaCl optimum (M)</th>
<th>k (error)</th>
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<th>Median plaque area/mm²</th>
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DISCUSSION

Palindrom-mediated inviability in an sbcC host is center-dependent in vivo: The evidence for the existence of DNA cruciforms in vitro has been comparatively lacking until recently (Panayotatos and Fontaine 1987; McClellan et al. 1990; Davis et al. 1991; Zheng et al. 1991; Sindel et al. 1991) and the pathway for extrusion
DNA Secondary Structure in E. coli

is only beginning to be investigated. Warren and Green (1985) demonstrated that large insertions of at least 50 bp in the center of a palindrome were required before inviability was overcome. This led them to argue that the structure responsible for inviability was dependent upon interactions between the palindrome arms and did not

FIGURE 4.—(A) Schematic illustration of the variation in plaque sizes for the AAD mutants. The size of the bar is proportional to the difference in plaque area compared with AAD-bke. AAD15C16C is dotted to make it easily visible. Mutations that decreased plaque size are shown above the line and mutations that increased it, below. Only mutations to the very central two base pairs had an effect in vivo that was predicted by the in vitro results under optimal salt conditions [see (B) and (C)], assuming that increased cruciform extrusion corresponds to decreased plaque size and vice versa. All other mutations altered the plaque size in a reverse manner compared with the in vitro data, the effect lessening further out from the center. (B, C and D) Schematic illustration of the variation in cruciform extrusion rates in vitro. The size of the bar is proportional to the change in rate for that sequence compared with the parent bke/pADbke. Mutations that enhanced the in vitro extrusion rate are shown above the line and mutations that depressed it are shown below the line. The results for 16C are overlaid and dotted so that they are easily visible. (B and C) Data from Murchie and Lilley (1987) and the pAD mutants, respectively, in the optimal salt (NaCl) buffer. Mutations to A/T base pairs tended to enhance extrusion and mutations to C/G had the reverse effect. Changes to the center had the greatest effect, although changes in the peripheral base sequence were not always insignificant [see 11T in (B) and 10G in (C)]. The same changes had similar effects in the two plasmid systems, although mutations to C/G pairs had a much greater relative effect in pMS2B. In both instances the 15C mutation caused extrusion to be unexpectedly fast. (D) Extrusion in buffer P (150 mM potassium glutamate/4 mM magnesium acetate) for the pAD mutants. This buffer was selected to mimic physiological conditions. For six out of the eight palindromes, cruciform extrusion in vitro was found to follow the same relative order compared with the plaque area data [evident from the similar shapes of (A) and (D)]. Plasmids pAD10G and pAD13G still extruded slowly compared with pADbke (A. Davison and D. R. F. Leach, unpublished observations).
involves the center. However, Chalker et al. (1993) were able to detect increased viability produced by much smaller asymmetries in long DNA palindromes and concluded that a center-dependent pathway for extrusion operates, and moreover, that it is independent of *E. coli* genotype for the strains studied. Zheng et al. (1991) came to a similar conclusion regarding the center-dependent using a physical assay for cruciforms, and Sinden et al. (1991) reported that the stability of base pairing in the hairpin stem and ease of cruciform formation affect the deletion frequency. T. Allers and D. R. F. Leach (unpublished) have recently demonstrated that long DNA palindromes in *λ* phage adopt a methylation-resistant structure that is consistent with the presence of cruciforms *in vivo*.

If a center-dependent pathway for the formation of a cruciform structure exists in *E. coli* then this predicts that palindrome-mediated inviability will be particularly sensitive to the base sequence at the center of the palindrome and progressively less sensitive to more peripheral sequence changes. The results reported here are consistent with this prediction; the data are presented in Table 1 and Figure 4.

**Intrastrand pairing is critical to the viability in *sbcC* hosts**: The plaque size of the *λ* phage was progressively less affected by changes further outside the center of the palindrome, implying that palindrome-mediated inviability is center-dependent. (this effect is evident from the triangular shape of Figure 4A). Such effects are consistent with the presence of cruciforms *in vivo*. However, the effects of base sequence changes on viability do not correlate with S-type extrusion kinetics at the optimal salt concentration *in vitro*. In fact, it was found that changing GC to AT base pairs (outside the central 2 bp) increased the plaque size, and vice versa. These results argue that intrastrand pairing between bases in the palindrome center, as opposed to central melting, is the major determinant of viability. The effects of sequence changes on viability can only be interpreted in terms of DNA melting for the central two base pairs. Thus, the *λ* phage with a central 5'-AT-3' or 5'-TA-3' sequence (AADbke, λAD16T) both have considerably smaller plaques than the comparable phage with a 5'-CG-3' sequence at the center (AAD16C, λ15C16C). Alternatively, the differences between these four phages could be explained in terms of altered loop structure and stability, rather than DNA melting. The fact that sequence changes up to 7 bp away from the center have an effect upon the plaque size (compare λAD10G with AADbke), suggests that the structure causing inviability may not be fully stabilized unless the stem is at least 5–6 bp. Whether or not cruciform structures are responsible for the observed effects on viability, the results argue strongly for a role of intrastrand base pairing at the palindrome center.

**Cruciform extrusion *in vitro* in a “physiological” buffer models *in vivo* behavior more closely**: Cruciform extrusion *in vitro* in a “physiological” buffer models the effects of long palindromes on λ viability more closely than S-type extrusion under optimal salt (NaCl) conditions. The extrusion reactions in buffer P suggest that, under these conditions, intrastrand base pairing (as opposed to DNA melting) becomes relatively more important in determining the rate of *in vitro* extrusion. That it is possible to partly model the viability by cruciform extrusion *in vitro* provides further evidence that a cruciform structure may be responsible for the effects measured *in vivo*. It is likely that the added magnesium is responsible for much of the altered extrusion kinetics observed since magnesium is known to specifically affect DNA supercoiling. Holliday junction structure, and branch migration. However, if palindrome-mediated inviability is due to the presence of cruciform structures *in vivo*, then it will be determined by both the process of getting there (cruciform extrusion) and staying there (cruciform stability). Clearly, the cruciform extrusion reactions model only part of this. Additionally, it is not possible to rule out the effect of protein-DNA interactions that might occur as a consequence of processes such as transcription, replication, or homologous recombination.

**DNA loop structures *in vivo* may contain only two residues**: Most information on DNA loops has come from studies of DNA hairpins *in vitro*. Early reports suggested that a loop of four residues was normal (Haasnoot et al. 1983; Hilbers et al. 1985), but recently several studies have found evidence for hairpin loops of only two residues in specific base sequences (Xodo et al. 1988; Bloemers et al. 1991; Kallick and Wemmer 1991; Raghunathan et al. 1991). Conclusions on loop size in the AAD cruciforms can be reached by comparing the plaque areas of AADbke (0.36 mm²), AAD15C (0.17 mm²), AAD16C (0.70 mm²), and λAD15C16C (0.46 mm²). AAD15C had the smallest plaques among the phages constructed, yet its extra CG base pairs were in positions that would be unpaired in a four residue cruciform loop, and should therefore not have affected intrastrand pairing to a great extent. Similarly,
AAD15C16C had CG base pairs at these same positions in place of the AT base pairs present in AAD16C. It is noted that the equivalent plasmid palindrome pAD15C (and the corresponding bke palindrome) also extruded unexpectedly rapidly in vitro. Furthermore, in buffer P, both pAD15C and pAD15C16C extruded particularly rapidly, relative to pADbke and pAD16C, respectively. Mürchle and Lilley (1987) suggested that this discrepancy in their data was due to the alternating purine/pyrimidine structure (present in pAD15C) reducing the DNA stability at the center of the inverted repeat. While this is a possibility, a more likely explanation of the severe effect in vivo is that the DNA loops of the cruciform structures formed in AAD15C and AAD15C16C contain two residues only (or were four base loops with some two base character). The data suggest that the extra CG pairs in AAD15C and AAD15C16C stabilize the loop structure in vivo, and therefore contribute to palindrome mediated inviability. This explanation is supported by the work of Blommers et al. (1989) who implied that the central sequence 5'-CATG-3' could theoretically form a two base loop in a DNA hairpin in vitro. Lowered loop stability could also explain why AAD16T (5'-ATAT-3') had only slightly smaller plaques than AADbke (5'-AATT-3') despite the pAD16T palindrome extruding more rapidly in vitro. The loop of AADbke could be more stable in vivo because the central sequence was 5'-AT-3' with the purine in the 5' position rather than the reverse (Blommers et al. 1989). A possibility that cannot be discounted is that the in vivo melting stability of dinucleotide base pairs are not identical to the values that have been determined in vitro. The observed differences between the palindrome mediated inviability and cruciform extrusion in vitro may not be surprising in view of the highly complex environment of the cell.

Implications for cruciform extrusion of naturally occurring palindromes in wild-type hosts: The results presented here demonstrate the existence of a center-dependent pathway of palindrome-mediated inviability in sbcC mutants, and therefore suggest that a pathway for cruciform formation exists in vivo. Chalker et al. (1993) suggested that cruciform extrusion of long palindromes is not dependent on the sbcC genotype and therefore that SbcC may act in the processing of cruciform structures rather than in their formation. This is supported by two other observations both arguing that sbcC may encode a nuclease. The first is that the gam gene of bacteriophage λ (known to encode a nuclease inhibitor) permits the propagation of long palindromes via an interaction with sbcC (Kulkarni and Stahl 1989). The second is that the SbcC polypeptide is distantly related to genes responsible for the major exonuclease activity of bacteriophages T4 and T5 on host chromosomal DNA, T4 gt-46 and T5 gpD13 (Leach et al. 1992). Furthermore, it has been shown that sbcC mutants do not have an altered level of intracellular DNA supercoiling (J. Lindsey and D. Leach, unpublished observations).

The implication of the results presented here is that a center-dependent pathway for intrastrand base pairing, consistent with cruciform extrusion, also exists for wild-type hosts. At a given threshold length or stability of intrastrand base pairing the structure may become a substrate for the SbcC and D proteins. The short range of the base sequence changes studied here argues that similar transient reactions are likely to occur in short, naturally occurring DNA palindromes. However, in these cases the equilibrium may normally be significantly in favor of the unextruded conformation for thermodynamic reasons.

The authors would like to thank Ewa Okely for expert technical assistance, Alison Chalker for some preliminary experiments, Chris Jeffery for assistance with the image analysis, Bob Lloyd for the bacterial strain, Gerry Smith for helpful discussions, and Richard Hatwood for critical reading of the manuscript. This work was supported by grants G5707935 CB and G9011717 CB from the Medical Research Council. A.D. is supported by a studentship from the Medical Research Council.

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Communicating editor: R. Maurer