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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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*. In addition, 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 replicated 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; SHURVINTON *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 (MURCHIE 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 unmelted during that step (MURCHIE and LILLEY 1987). Whether cruciforms exist *in vivo* has been in dispute until relatively 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 intra-strand 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.

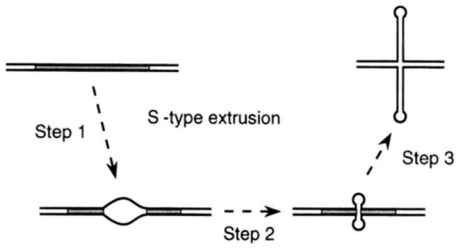

FIGURE 1.—S-type pathway for cruciform extrusion *in vitro* adapted from MURCHIE *et al.* (1992). There is an initial melting of central base pairs (step 1), followed by intrastrand pairing and protocruciform formation (step 2), then finally branch migration to the fully extruded cruciform (step 3). The transition state is probably the central melted bubble or the protocruciform. Central base opening is believed to involve about 8–10 bp. The whole process is driven by negative supercoiling.

MATERIALS AND METHODS

Media, bacterial strains, bacteriophage strains and plasmid vectors: Casitone-agar (CA) medium contained 10 g Bactocasitone, 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°, and to a volume of exactly 50 ml. λ 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 N2364 (AB1157 *sbcC*201 *phoR*::Tn*10*), and was obtained from R. G. LLOYD. The palindrome containing phages were all derived from DRL133 λ *pal spi*6 *cI*857 (CHALKER *et al.* 1993). DRL133 was crossed with DRL152 λ*spi*6 *cI*857 χ*C*153 to introduce a Chi site. The resultant phage DRL167 λ*spi*6 *cI*857 χ*C*153 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 palindrome in DRL167 is a 462-bp perfect inverted repeat, flanked by two *Eco*RI sites and with a unique central *Sac*I site. The *Sac*I 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 *Sac*I 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 palindrome 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 pAT153 palindromes used by MURCHIE and LILLEY (1987).

The center of each palindrome was subcloned and sequenced to confirm that it was as predicted. The palindrome was gel purified after restriction with *Eco*RI (or *Ava*I and *Pvu*I for the palindromes with an *Eco*RI center). A 32-bp *Taq*I fragment containing the palindrome center was then ligated into the *Acc*I 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 λAD phage (*e.g.*, pADbke and pAD16T). All manipulations used standard methods, as detailed in SAMBROOK *et al.* (1989).

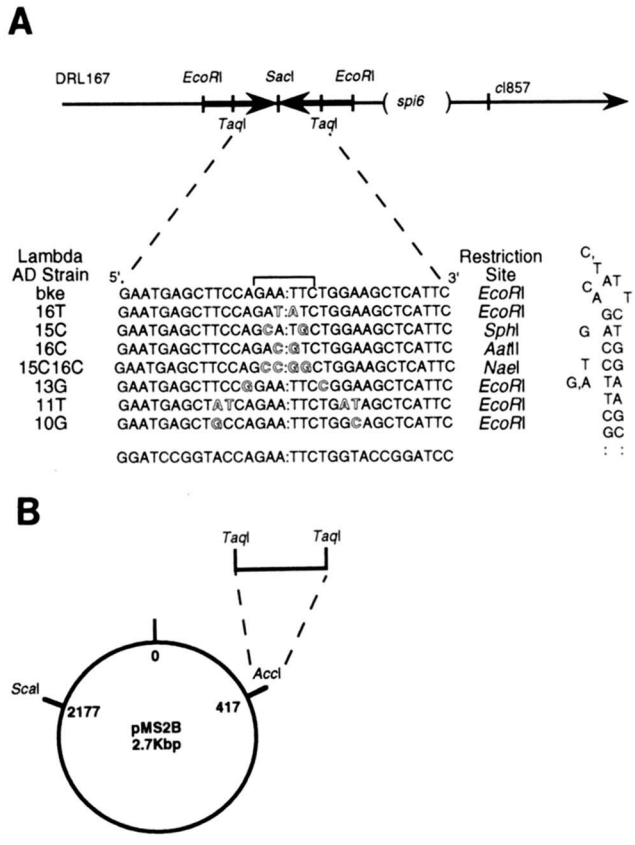

FIGURE 2.—Bacteriophage λ and plasmid constructs. (A) Top: partial map of DRL167. The full length of the palindrome *Eco*RI restriction fragment is 454 bp. Bottom: the structure and derivation of the palindromes studied, showing part of the potential cruciform structure on the right. The top sequence λADbke is considered the parent and differences from it are highlighted as open letters. To ease comparisons, each λ was named similarly to the equivalent MURCHIE and LILLEY (1987) construct in pAT153. At the bottom is the parent sequence that MURCHIE and LILLEY (1987) employed and it is presented for comparison. (B) The structure of pMS2B, a derivative of pUC18. *Taq*I restriction fragments containing the center of the palindrome were subcloned into the *Acc*I site of pMS2B. The resultant plasmids were named similarly to their λ parents (pADbke, pAD16T, *etc*).

Bacteriophage plating: An overnight culture of *E. coli sbcC* was diluted 1:10 in L-broth supplemented with 5 mM $MgSO_4$/0.2% maltose and grown for 140 min at 37°. An equal volume of 10 mM Tris-HCl (pH 8), 10 mM $MgSO_4$ was added. The diluted cultures were stored at 4° and used within 3 days. Aliquots of 0.25 ml of the cell suspension were incubated with a suitable dilution of phage at 37° 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°, the area of individual plaques on the cell lawn was measured using a Quantimet 970 Image Analyser (C. JEFFREE, 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 endonuclease 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 butan-1-ol extraction on ice, followed by extensive dialysis in 10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA at 6°. The plasmid DNA was stored at −70° and thawed slowly at 6° 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 extrusion 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° (±0.01) in a Grant LTD6 waterbath. The samples were then diluted into T4 endoVII reaction buffer (0.5 M Tris-HCl pH 8.0, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 5 mM spermidine, 250 μg/ml bovine serum albumin; PICKSLEY *et al.* 1990) and incubated with 40 units T4 endoVII at 16° for 40 min. No cruciform extrusion was detectable in this buffer at 16°. After this, the DNA samples were ethanol precipitated, resuspended in a suitable restriction buffer, and digested to completion with *Sca*I. 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 negative with a Shimadzu densitometer. A graph of 1 + ln (unextruded fraction) against time was plotted using linear regression analysis. The reaction constant (k) was calculated from the gradient of the graph and the reaction half-time as ln $2/k$.

Molecular biological procedures: To obtain DNA adequate for sequencing, QIAGEN plasmid "midi-prep" kits were used according to the manufacturer's instructions (QIAGEN Inc., Chatsworth, California). To recover DNA fragments, the DNA was purified from agarose gels following the procedure recommended using Gene Clean kits (BIO 101, Inc.). Restriction enzymes and DNA ligase were used according to the manufacturers' instructions. DNA sequencing was performed with the Sequenase kit v2.0 (U.S. Biochemical Corp.) and as described by the suppliers, except that the DNA was denatured by boiling and cooling rapidly in dry ice. Primer annealing took place at room temperature during the labeling step and the extension reactions were run at 45° 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* (MURCHIE 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.

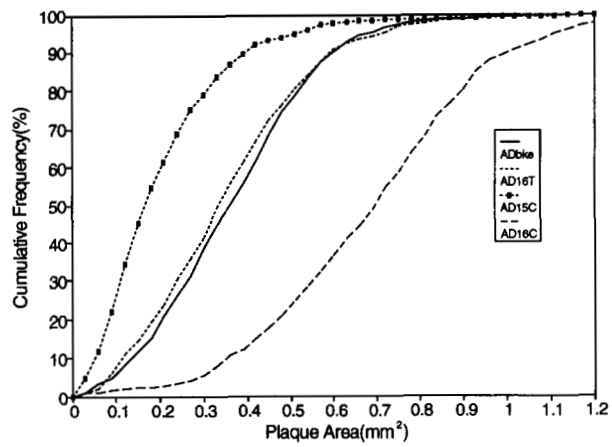

FIGURE 3.—Cumulative frequency distribution of plaque sizes for four of the λ phages. The differences between λAD15C, λADbke, and λAD16C were clearly visible to the eye, but the difference between λADbke and λAD16T was only visible after quantification by image analysis. The median was taken as the point at which the 50% cumulative frequency intersected the line on the *x*-axis.

Plating behavior of palindrome phage: The phenotype conferred upon the λ by the palindrome was assessed by plating on a lawn of N2364 *sbcC*201, 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

TABLE 1

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

	Cruciform extrusion											
	bke series in pAT153, (NaCl buffer) from MURCHIE and LILLEY (1987)				pAD series (NaCl buffer)				pAD series (buffer P)			
Mutant	k(error)	Exp	$T_{1/2}$ (min)	NaCl optimum (MM)	k(error)	Exp	$T_{1/2}$ (min)	NaCl optimum (MM)	k(error)	Exp	$T_{1/2}$ (min)	Median plaque area/mm^2
bke	−1.7 (0.02)	−4	70	50	−1.9 (0.34)	−3	6.1	45	−1.3 (0.17)	−3	8.9	0.36
16T	−1.3 (0.14)	−3	9	50	−4.0 (0.41)	−3	2.9	45	−2.1 (0.48)	−3	5.5	0.33
15C	−1.7 (0.04)	−4	67	75	−2.0 (0.26)	−3	5.8	45	−2.1 (0.29)	−3	5.5	0.17
16C	−1.7 (0.18)	−5	692	50	−1.9 (0.20)	−5	608	45	−5.2 (0.78)	−6	2222	0.70
15C16C	−7.0 (0.60)	−6	1649	50	−1.2 (0.14)	−5	963	45	−6.9 (0.69)	−6	1674	0.46
13G	−1.0 (0.09)	−4	114	75	−8.9 (0.15)	−4	13.0	60	ND			0.24
11T	−1.5 (0.07)	−3	8	50	−4.8 (0.53)	−3	2.4	45	−1.2 (0.12)	−3	9.6	0.41
10G	−1.4 (0.12)	−4	81	50	−2.4 (0.26)	−4	48.1	45	ND			0.33

Rate constants are expressed as $k(\pm\text{standard error}) \times 10^{\text{exp}}$. Half-times were calculated from ln $2/k$. ND, k not determined.

λAD13G, but smaller plaques than λADbke. Significantly, this pattern also included λAD15C and λAD15C16C. Clearly, however, the precise determinants of the degree of inviability of a palindrome-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 λ phage with a 5′-AT-3′ or a 5′-TA-3′ at the very center of the palindrome (λADbke and λAD16T, plaque sizes 0.36 and 0.33 mm^2) had smaller plaques than the two phage with 5′-CG-3′ at the center (λAD16C, plaque size 0.70 mm^2; λAD15C16C, plaque size 0.46 mm^2), consistent with greater levels of the structure causing inviability for the former λ phage. For changes outside the central two base pairs the direction of change was the reverse of that predicted by the S-type model applicable *in vitro*, implying that the intrastrand pairing between the arms of a palindrome may be critical in determining the level of inviability. Changes that *slowed in vitro* extrusion *decreased* the plaque size, and *vice versa*. For instance, λAD13G (central sequence **GGAATTCC**) had smaller plaques than λADbke (central sequence AGAATTCT).

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 *Taq*I fragment containing the λ palindrome center was subcloned into the *Acc*I 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 centers of MURCHIE and LILLEY (1987) in pAT153.

The cruciform extrusion salt optima that were determined for the pAD series are indicated alongside the equivalent bke series salt optima in Table 1. For most of the reactions the salt optimum was found to be 45 mM, compared with 50 mM in the experiments of MURCHIE and LILLEY (1987). For the two bke sequences that had a raised salt optimum of 75 mM (15C and 13G), only one of the pAD equivalents had a similarly raised optimum (pAD13G). To determine the reaction constant a time course was performed at the optimum salt concentration. A sample extrusion time course experiment is illustrated in Figure 5. The extrusion data are detailed in Table 1 and further compared with those of MURCHIE and LILLEY (1987) in Figure 4, B and C. In general, the changes that were made to the palindromes had comparable effects in both vectors with one exception: pAD10G extruded more slowly than expected, relative to pADbke.

Cruciform extrusion *in vitro* in a "physiological" buffer: Cruciform extrusion of the pAD series of palindromes was carried out in a more physiological buffer (buffer P; containing potassium glutamate and magnesium acetate, suggested by G. SMITH). The extrusion half times that were determined are detailed in Table 1 and compared with the plaque assay results in Figure 4, A and D. It was found that buffer P had a significant effect upon the relative extrusion kinetics of the palindromes, and that the reaction half times for six out of the eight plasmids could be compared to the equivalent order of plaque areas. The relative reaction constants for the two other plasmids remained relatively constant in this buffer (A. DAVISON and D. R. F. LEACH, unpublished observations).

DISCUSSION

Palindrome-mediated inviability in an *sbcC* host is center-dependent *in vivo*: The evidence for the existence of DNA cruciforms *in vivo* has been comparatively lacking until recently (PANAYOTATOS and FONTAINE 1987; MCCLELLAN *et al.* 1990; DAYN *et al.* 1991; ZHENG *et al.* 1991; SINDEN *et al.* 1991) and the pathway for extrusion

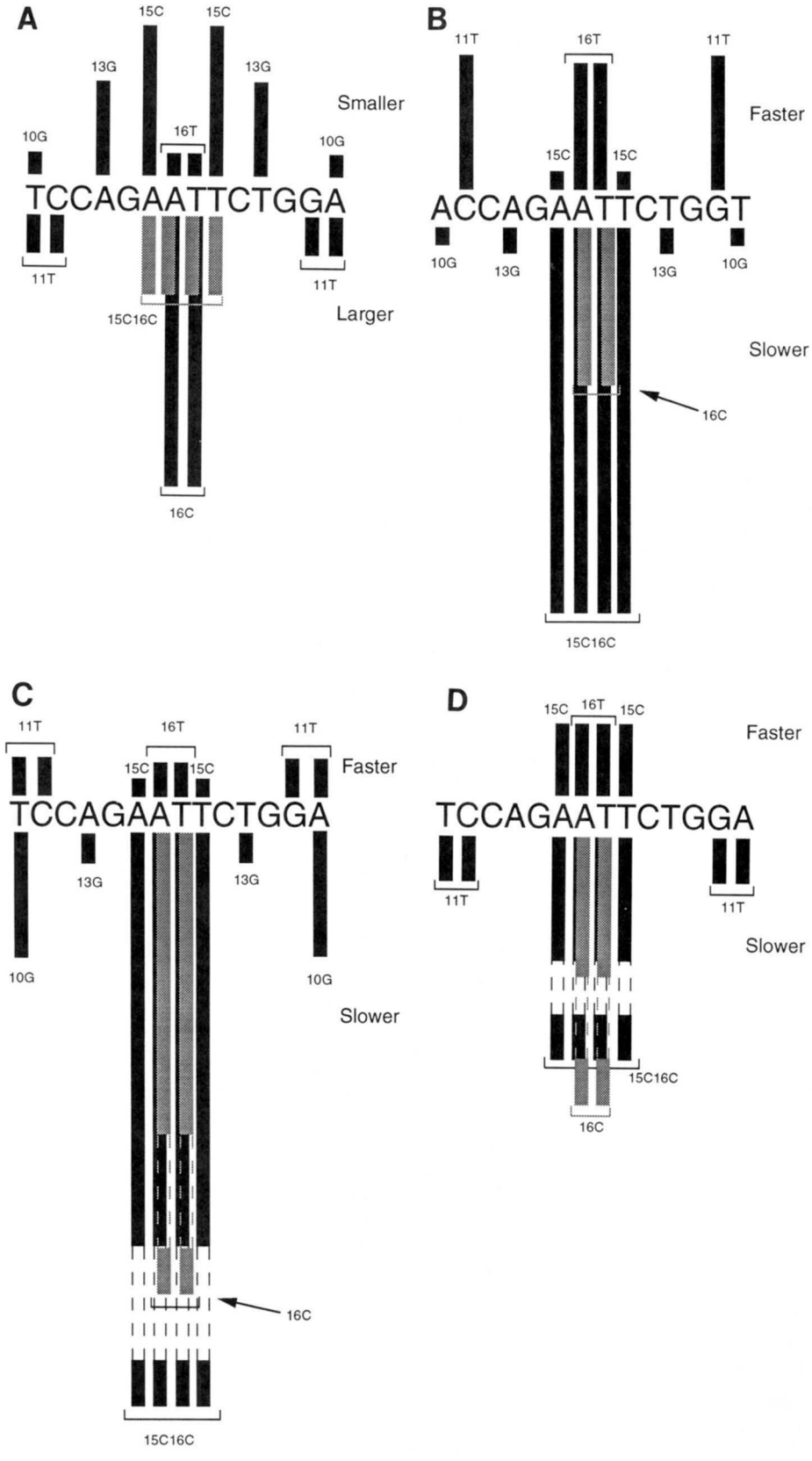

FIGURE 4.—(A) Schematic illustration of the variation in plaque sizes for the λAD mutants. The size of the bar is proportional to the difference in plaque area compared with λAD-bke. λAD15C16C is dotted to make it easily visible. Mutations that decreased plaque size are shown above the line and mutations 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).

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

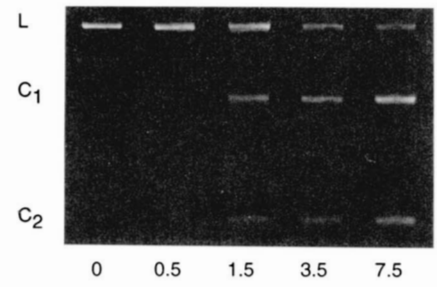

FIGURE 5.—An example of cruciform extrusion at 37°. pAD11T was incubated in 45 mM NaCl, 10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, and sampled between 0 and 7.5 min as shown. Full length linear plasmid DNA is indicated by the letter L. The fragments resulting from T4 endoVII cleavage at the cruciform are labeled C_1 and C_2.

involve 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-dependence 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 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 (λADbke, λAD16T) both have considerably smaller plaques than the comparable phage with a 5′-CG-3′ sequence at the center (λAD16C, 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 λADbke), 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 viabilty 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 effect 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; BLOMMERS *et al.* 1991; KALLICK and WEMMER 1991; RAGHUNATHAN *et al.* 1991). Conclusions on loop size in the λAD cruciforms can be reached by comparing the plaque areas of λADbke (0.36 mm^2), λAD15C (0.17 mm^2), λAD16C (0.70 mm^2), and λAD15C16C (0.46 mm^2). λAD15C 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,

λAD15C16C had CG base pairs at these same positions in place of the AT base pairs present in λAD16C. 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. MURCHIE and LILLEY (1987) suggested that this discrepency 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 λAD15C and λAD15C16C contain two residues only (or were four base loops with some two base character). The data suggest that the extra CG pairs in λAD15C and λAD15C16C 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 λAD16T (5′-ATAT-3′) had only slightly smaller plaques than λADbke (5′-AATT-3′) despite the pAD16T palindrome extruding more rapidly *in vitro*. The loop of λADbke 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 stabilities 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 *gp46* and T5 gp*D13* (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 occuring DNA palindromes. However, in these cases the equilibrium may normally be significantly in favor of the unextruded conformation for thermodynamic reasons.

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