Left-handed DNA

CLONING, CHARACTERIZATION, AND INSTABILITY OF INSERTS CONTAINING DIFFERENT LENGTHS OF (dC-dG) IN ESCHERICHIA COLI

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Recombinant pBR322 derivatives were constructed containing tracts of (dC-dG) sequences which are 58, 32, 26, and 10 base pairs (bp) in length in conjunction with a 95-bp fragment containing the Escherichia coli lac operator-promoter. The biological properties of these plasmids were unusual since deletions in the (dC-dG) regions, but not in the pBR322 nor the lac segments, were frequently observed; segments of (dC-dG) longer than approximately 50 bp were not stable but suffered deletions. Segments of approximately 30 bp or shorter were stable in most cases. The (dC-dG) tracts seemed to enhance recA-mediated recombination when they were of suitable length and were cloned into certain sites in the recombinants. Also the (dC-dG)-containing plasmids were less supercoiled (by 6-12 turns) than expected, relative to control plasmids, after isolation from E. coli hosts. These recombinant plasmids were used in the following paper to evaluate the properties of segments containing the unorthodox left-handed conformations.

The role of DNA structure in gene regulation (reviewed in Refs. 1 and 2) is a subject of growing interest due to the availability of new types of probes. Left-handed DNA is one of several unusual conformations and could provide a variety of target sites for protein interactions with DNA segments which are not present in right-handed helices. The recent demonstration (3-6) that left-handed DNA can exist in recombinant plasmids and restriction fragments provides substantial support for these notions. Since left-handed conformations existed in close proximity with right-handed helices, a conformational junction must exist in these molecules. Furthermore, the methylation of dC residues in (dG-dC), (7) strongly facilitated the salt-induced conversion from a right-handed to a left-handed helix (8, 9). This observation is significant since DNA methylation may be a regulatory process (10).

The unusual Z-type left-handed conformation was demonstrated by x-ray crystallography on tetramers and hexamers of (dC-dG) (11 and reviewed in Refs. 1 and 2); phosphorus NMR (12), proton NMR (13), Raman spectroscopy (14, 15), and CD (16, 17) showed that left-handed structures can exist for polymers of this DNA in solution. Left-handed DNA is not a new concept since this unusual type of helix was suggested in 1970 for (dI-dC), (18). The biological properties of a left-handed helix can be transmitted under a variety of environmental conditions (4). Also, these studies (4) revealed that a variety of left-handed conformations exist for (dC-dG) segments. Furthermore, the conformational properties of a left-handed helix can be transmitted through the junction to a neighboring DNA segment which has a modified DNA-B structure (5).

We wished to clone (dC-dG) segments of different lengths to evaluate the effect of helical length and properties on the B to Z transition. This manuscript describes the construction and characterization of pBR322 derivatives which contain (dC-dG) tracts. In characterizing these molecules, it was realized that their replication properties differed from all molecules cloned previously in this laboratory since deletions were commonly observed. The second manuscript (19) evaluates the energetic inter-relationships between DNA supercoiling and the B-Z transition in these plasmids. The third manuscript (20) in this series describes the capacity of another DNA sequence (dT-dG), (dC-dA), to adopt left-handed conformations.

MATERIALS AND METHODS

Chemicals, DNAs, and Enzymes—Hpa II, Hha I, and the Micrococcus luteus DNA polymerase were prepared in this laboratory by established procedures (21, 22). All other enzymes were purchased from Bethesda Research Laboratories. 3-Bromo-4-chloro-3-indolyl-β-D-galactoside was purchased from Bachem Corporation. All other reagents were obtained from Sigma.

The 95-bp lac fragment containing the lac operator and promoter which had Eco RI ends (23) was a gift of W. Hillen (formerly this laboratory). The 203-bp lac fragment also containing these regulatory sites which had Eco RI ends (24) was a gift of S. C. Hardies (formerly this laboratory). Poly(dG-dC) was synthesized as described previously (7).

Recovery of DNA from Agarose Gel Electrophoresis—DNA fragments were recovered from agarose gels using a modification of the DEAE-paper procedure described previously (25). After gel electrophoresis, the bands were stained with ethidium bromide. Incisions made were above and below the DNA band of interest. DEAE-cellulose strips were inserted into the incisions in the gel. Electrophoresis then was continued until the DNA band had migrated into the paper strip (as visualized under UV light). The paper strip on the anode side of the desired band was placed into 1 ml of 1 M NaCl and was kept at 55 °C for 1 h. The extract was filtered through siliconized glass wool and was extracted with 0.5 ml of phenol (saturated with 0.2 M Tris-HCl buffer (pH 8.0)). After mixing, the suspension was kept at 0 °C for 10 min, and the phases were separated by centrifugation in an Eppendorf microcentrifuge. An agarose-containing inter-
face was observed between the aqueous and phenol phases. The aqueous phase was transferred to another tube and the phenol extraction was repeated several times until no agarose was observed at the interface. The aqueous phase then was extracted 3 times with an equal volume of ether and the DNA was precipitated with 3 volumes of ethanol. The pellet was rinsed with 80% ethanol and dried in vacuo. The DNA was dissolved in a small volume of 10 mM Tris-HCl buffer (pH 8.0). Recovery of DNA was approximately 60%.

**Other Methods**—The reaction conditions for digestion with restriction enzymes and for gel electrophoresis (24, 26) were described previously. Reaction conditions for filling in the sticky ends of restriction enzymes were described as reported (24). DNA sequencing procedures which were described previously (24) were used for selection of candidates containing the lac operator in the inserted fragment. The mini-prep procedure for screening a large number of candidates for insertions (29) was employed for isolation of plasmid DNA. Also, plasmids were isolated by procedures which were described previously (24). DNA sequencing was performed by the Maxam and Gilbert method (30). E. coli MO recA was a gift from L. Wray and W. Reznikoff (this department). All other methods were as described previously (6, 24).

**RESULTS**

**Cloning of (dC-dG) in pBR322**—The strategy for cloning (dC-dG) sequences is outlined in Fig. 1. We wished to clone a family of molecules containing the lac operator-promoter on a 95-bp fragment which is flanked by (dC-dG) sequences. Our strategy was designed to regenerate single Bam HI sites on both ends of the inserts to facilitate isolation of the insert from the pBR322 vector. Because the lac 95-bp fragment includes the operator, recombinant molecules can be screened easily in vivo for lac constitutivity (24, 27) by selecting blue colonies on 5-bromo-4-chloro-3-indolyl-β-D-galactoside plates.

Poly(dG-dC)-poly(dC-dG) (7) was cleaved with Fnu DII to oligomers with an average length of approximately 600 bp as judged by electrophoresis on 2% agarose gels. This enzyme was chosen since it generates blunt ends which would be suitable for subsequent ligation. Furthermore, a guanosine residue at the 3’ end should permit regenerations by DNA polymerase. This procedure should have caused the 32 bp of (dC-dG) residues on one end of the fragments; the other DNA polymer to restriction fragment was employed in order to prevent oligomer formation for the (dC-dG) fragments. A molar ratio of 10:1 of Bam HI and the sticky ends filled-in vector. After removal of Fnu DII by phenol extraction, the oligomers were ligated to the Alu I blunt ends of the 95-bp lac fragment. A molar ratio of 10:1 of the DNA polymer to restriction fragment was employed in order to prevent oligomer formation for the lac 95-bp fragment. To further eliminate the polymerization of the lac 95-bp fragment, Alu I was added to the ligation reaction.

Since our goal was to clone the lac 95-bp fragment which is flanked by (dC-dG) oligomers, another purification step was employed to reduce the background of inserts of the lac 95-bp fragment which do not contain the DNA polymers. The ligation products were fractionated on 2% agarose gels and the lac 95-bp fragment was approximately 600 bp. It is clear that a powerful selection allowed the lac 95-bp region. However, the fragments identified as Group A and Group B are heterogeneous in the length of the (dC-dG) residues on one end of the fragments; the lac 95-bp region and the 32 bp of (dC-dG) are homogenous in these groups.

**Bam HI site, all contained inserts and had three interesting features. (a) The Bam HI site was always distal to the Eco HI site (in the pBR322 vector) as revealed by a double digest with Eco RI and Bam HI. (b) All inserts were 200 bp or shorter in length. Since the average length of (dC-dG) oligomers which were ligated to the lac 95-bp fragment was approximately 600 bp, it is clear that a powerful selection allowed only the shorter (dC-dG) tracts to be cloned. (c) Some plasmid preparations contained two inserts of different sizes as shown by digestion with both Eco RI and Bam HI. Digestion of some of these double inserts with Fnu DII revealed the presence of the lac 95-bp fragment. Thus, these inserts contained (dC-dG) sequences on both ends of the lac 95-bp fragment. These results strongly suggest that the variation in the size of the inserts was due to differences in the lengths of (dC-dG) tracts which flank the lac 95-bp fragment.

**Characterization of the pRW750 Class of Plasmids**—The plasmids (pRW750) containing both Bam HI sites were very...
unusual since they contained inserts of different lengths. When the pH750 class of plasmids was digested with Bam HI, fragments of several sizes were observed (Fig. 2, Lane 1). The longest insert was a sharp band corresponding to 187 bp, but the two shorter groups of fragments were smeared; the latter two groups of fragments were designated Group A and Group B and their sequence features are shown in Fig. 1. These groups of fragments represent families of inserts of different sizes. The fragments from these three bands were isolated from the gel and were re-electrophoresed (Fig. 2, Lanes 2–4). Again, it can be seen that the 187-bp fragment is homogeneous whereas the fragments in Group A and Group B are classes of molecules.

Each of these three bands was digested with Hpa II, and gel electrophoresis (Fig. 2, Lanes 5–7) showed that all three digests contained a single discrete band 91 bp in length. However, a second band was observed in each case. The 187-bp fragment gave a single sharp band at 96 bp in addition to the 91-bp fragment. However, the Group A and Group B fragments each gave a smear of fragments of different and smaller sizes (Lanes 6 and 7, respectively) in addition to the 91-bp fragment.

Furthermore, when each of the fragments shown in Lanes 2–4 was digested with Fnu DII, which totally digests the (dC-dG) regions, but no sites are present in the lac 95-bp segment, bands co-migrating with the lac 95-bp fragment were the only visible products on acrylamide gel electrophoresis (Fig. 2, Lanes 8–10).

In summary, all three sets of inserts in this plasmid are closely related and contain the same 91-bp sequence from one of the Bam HI sites to the centrally located Hpa II site. However, the other end of each of these groups of fragments varies in size (as illustrated on the bottom of Fig. 1) and the variation occurs only in the (dC-dG) tract, not in the lac 95-bp segment.

To evaluate this unusual behavior more rigorously, DNA sequence studies were performed on the 187-bp fragment and on the bands corresponding to Group A and Group B. DNA sequencing (30) showed that the 187-bp fragment had the sequence shown in Fig. 1 with the lac 95-bp sequence flanked by 56 bp of (dC-dG) on one side and 32 bp of (dC-dG) on the other side. Furthermore, sequence studies revealed that the Group A and Group B fragments both had perfect sequence identity with the 187-bp fragment in the 91-bp long segment from the Bam HI site to the Hpa II site. However, the other end of the fragment was heterogeneous in sequence since the gel could only be read to a limited extent (approximately 10–15 bp). This result confirms the sequence heterogeneity described above in one end of the Group A and Group B fragments; these sequences are illustrated on the bottom of Fig. 1.

This sequence heterogeneity only can be explained by deletion events in vivo, and it seems likely that the deleted fragments (Group A and Group B) are derived from the 187-bp sequence. The (dC-dG) arm which is 32 bp in length was stable in all cases, whereas the arm which is 56 bp in length was not stable but suffered deletions.

The Deletion Phenomenon—This observation that a clone contained a family of inserts varying in length in only one of the (dC-dG) segments was very unexpected. In an attempt to evaluate the factors involved in this behavior, we tried to reclone the purified 187-bp fragment (Fig. 2, Lane 2) into pBR322. The recombinant plasmids which were isolated were digested with Bam HI to release the insert. Fig. 3 shows typical results: all plasmids contained the 187-bp fragment as well as multiple molecules of small size (Lanes 2–6) which range in size from 150 to 187 bp in length. In all cases (of approximately 30 isolates), we found a heterogeneous population of plasmid molecules. The parent recombinant molecule was transformed into recA’ BC- as well as recA’ BC+ cells. A family of inserts was found in all cases. This suggests that the deletion events did not depend on the products of these recombination genes.

The deletion patterns were monitored through six sequential growths (each of 4-h duration) of E. coli harboring pH750. The plasmid containing multiple length inserts was maintained. In some cases, inserts formed discrete bands with a much lower content of 187-bp fragment.

Construction and Characterization of pH751—One of the goals of this work was to obtain a single recombinant molecule containing (dC-dG) tracts flanking the lac 95-bp insert in order to perform a variety of biochemical and physical studies (1, 3–6) on the role of left-handed DNA in natural systems. Since the above results inferred that the deletion of (dC-dG) segments was dependent on the length of the (dC-dG) tract, it should be possible to reclone one of the deleted fragments and stably maintain it in E. coli. Accordingly, we excised a group of fragments of approximately 160 bp in size from a gel similar to that shown in Fig. 3, Lane 1, and cloned this class of molecules into pBR322. One of the clones contained an insert 157 bp in length with the lac 95-bp sequence flanked by 32 and 26 bp of perfectly alternating (dC-dG) regions. The identity of this insert between the Bam HI sites was revealed by DNA sequence analysis (30). A preliminary description of pH751, the 157-bp insert, as well as several other fragments derived from the 157-bp insert, was reported previously (3).

pH751 has been prepared repeatedly by a number of investigators in this laboratory in cultures ranging from several milliliters to 300 liters over a period of at least 1 year. In all cases, the 32- and 26-bp (dC-dG) regions (as well as all other DNA sequences) were stable and did not suffer deletions. This result further emphasizes the relationship of the length of the (dC-dG) regions to the deletion phenomena.
Cloning of Oligomers of 157- and 187-bp Fragments—Attempts were made to clone oligomers of the 157- and the 187-bp fragments in order to further evaluate the effect of length of (dC-dG) tracts on the deletion phenomena as well as to attempt to clone tandem inserts for the purpose of large scale preparation of the fragments. Thus, the purified 187-bp fragment (and the 157-bp fragment in a parallel experiment) was treated with T4 DNA ligase; the products of each of these reactions showed a ladder representing the family of ligated products, as expected from previous studies (23, 31). These mixtures of oligomers then were ligated with Bam HI, and the mixtures were used to transform E. coli C600 SF8.

Thus, the oligomers of the 187-bp fragment (and of the 157-bp fragment) should retain tracts of (dC-dG) which are internal to the lac 95-bp sequences. Also, the lengths of the (dC-dG) regions should be the sum of the length of the arms of the (dC-dG) regions from the 187-bp (and the 157-bp) monomers. Of course, a number of combinations of the (dC-dG) tracts is possible, but the shortest length should be 52 bp which contains a duplex GATC interruption in the center due to the ligation of the Bam HI sticky ends.

The transformants frequently contained plasmids with a variety of lengths of insertions between the Bam HI sites. In many cases, insertions which did not correspond to the size of the monomers were found, but were longer than 187 bp (or 157 bp). In order to evaluate the composition of these inserts, some of the plasmids were digested with Bam HI and the inserts which were isolated were cleaved with Fnu DII. In all cases, an intact 95-bp lac fragment was observed by polyacrylamide gel electrophoresis. Again, this indicates that the lac 95-bp fragment was not involved in the deletion events but that deletion occurred in the (dC-dG) sequences as well as at the duplex Bam HI (GATC) site. Thus, we conclude that the deletion is a function of the length of the (dC-dG) tracts even if they are interrupted by GATC. Furthermore, it has not been possible to successfully prepare tandem repeat inserts, as done previously (23, 31) for other types of fragments for the purpose of large scale isolation.

Construction and Characterization of pRW755, pRW756, and pRW757—Previous studies (3, 4, 6) revealed that it was possible to study the B to Z transition in a small segment of supercoiled molecules. This is the basis for a very sensitive assay for evaluating the number of base pairs involved in the transition and thus deducing the size of junction regions between right-handed and left-handed segments (1). Because of our capacity (3) to resolve topoisomers with different superhelical density on agarose gels containing high salt, we wished to evaluate the length of (dC-dG) tracts in a plasmid on the B to Z transition. Thus, the recombinants pRW755, 756, and 757 containing (dC-dG) tracts which were 32, 26, and 10 bp in length, respectively, were constructed and characterized.

Fig. 4 shows the strategy employed for the construction of pRW755 and pRW756. The 157-bp fragment containing the 32- and 26-bp lengths of (dC-dG) was cleaved with Hpa II. The two resulting fragments were separated by polyacrylamide gel electrophoresis and each was mixed then with the “other half” of the lac 95-bp fragment, which was also obtained by Hpa II cleavage. After ligation with the appropriate vector and transformation into E. coli C600 SF8, the desired plasmids were found.

pRW757 (Fig. 5) was prepared by taking advantage of the deletion phenomena. The class of recombinant molecules called pRW750 was digested with Bam HI to give the family

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**Fig. 3.** Electrophoretic analysis of inserts found after re-cloning of the 187-bp fragment in pBR322. The 187-bp fragment (Fig. 2, Lane 2) was cloned by the techniques previously described (24). The mini-prep DNA (29) was digested with Bam HI and the products were analyzed on 6% polyacrylamide gels. Lane 1 shows a control of pRW750 digested with Bam HI. Lanes 2-6 show the digests observed with five other plasmid preparations. Other details are described under “Materials and Methods.”

**Fig. 4.** Strategy of construction of pRW755 and pRW756. The preparation and characterization of the lac 95-bp fragment with Eco HI ends (23) and the 157-bp fragment with Bam HI ends (3) were described. Other details are under “Materials and Methods.”
of inserts shown in Fig. 2, Lane 1. The segment of the gel which corresponded to 125-140 bp was extracted, although no DNA was detectable at this position on the patterns. These putative fragments were ligated into the Bam HI site of pBR322 and numerous blue colonies were found after transformation. After isolation of the plasmid from an appropriate colony and digestion of the DNA with Bam HI, a 129-bp fragment was recovered and mixed with the 69-bp fragment containing lac 55 (see Fig. 4), and the mixtures were ligated into pBR322 which had been linearized by Eco RI and Bam HI digestion. The reason for this unusual behavior is uncertain but clearly demonstrates the marked influence in vivo of the (dC-dG) tracts. This influence may be (a) on the DNA gyrase and nick-closing activities that determine the superhelical density. For 10 plasmid preparations of pRW751, the plasmid always had a lower superhelical density than pRW451 (a plasmid of similar sequence and size (3) but lacking the (dC-dG) tracts) isolated from the same host strain. On average, pRW751 had 6-12 fewer supercoils than pRW451, as determined by electrophoresis on gels containing ethidium bromide (32). pRW755 and pRW756 as isolated also were less supercoiled than pRZ4032, an identical plasmid but containing no (dC-dG) tracts. pRW757 appeared to have a superhelical density similar to pRZ4032. These observations for pRW755, 756, and 757 are based on a limited number of plasmid isolations.

The reason for this unusual behavior is uncertain but clearly demonstrates the marked influence in vivo of the (dC-dG) tracts. This influence may be (a) on the DNA gyrase and nick-closing activities that determine the superhelical density of the plasmid in vivo, (b) a manifestation of DNA conformational changes which occur in certain DNA segments during plasmid isolation, or (c) due to unusual recombination (see above) or replication events. The in vitro synthesis of (dC-dG), - (dC-dG)3, (7, 33) was very unusual relative to 13 other DNA polymers (34) since only linear, not exponential, kinetics was found under a variety of conditions.

**DISCUSSION**

This paper describes the construction of several plasmids containing different lengths of (dC-dG) sequences. pRW751 was a pBR322 derivative containing a 157-bp Bam HI insert.

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2 R. D. Klein and R. D. Wells, unpublished observations.
comprising (dC-dG) sequences which were 32 and 26 bp in length that flanked the lac 95-bp fragment. pRW755 was constructed by replacing the Eco RI-Bam HI sequence of pBR322 by a fragment 125 bp in length composed of the lac 95-bp sequence and 26 bp of (dC-dG). pRW756 was identical to pRW755 except that it contained a longer (dC-dG) tract (32 bp in length), and the orientation of the lac fragment was reversed compared to pRW756. pRW757 was identical to pRW756 except that the (dC-dG) sequence was only 10 bp in length.

These plasmids and their inserts were constructed for the purpose of further evaluating the properties of left-handed DNA in recombinant plasmids and restriction fragments. Previous studies (3–8) demonstrated that (dC-dG) regions could be converted to left-handed helices whereas the neighboring natural sequences remained in right-handed structures. Thus, these molecules are suitable for the study of the B-Z junction, the energy inter-relationships between DNA supercoiling, and the B-Z interconversion (3, 19), as well as the interaction of the E. coli repressor and RNA polymerase with the operator and promoter in the lac-controlling elements which neighbor the left-handed conformations.

The cloning of (dC-dG) regions was very unusual compared to the successful cloning of a number of other DNA polymers (dA)n, (dT)n, (dG)n, (dC)n, triple-block polymers) in this laboratory (35) since deletions were commonly observed in the (dC-dG) segments. Attempts to clone long regions of (dC-dG) generated pRW750, which is a class of recombinant molecules that probably exists in a single E. coli cell; this class of molecules contained a 187-bp Bam HI insert and a large number of shorter inserts which were identical but had variable length (dC-dG) regions. The longest (dC-dG) tract which was cloned (56 bp) was not stable. However, when inserts were characterized which contained (dC-dG) blocks which were 32 bp in length or shorter, acrylamide gel analysis and sequence studies revealed no deletions. (This general rule, however, was violated for pRW757, since it was derived by (dA), (dT),, (dG),, (dC),, triple-block polymers) in this laboratory (35) since deletions were commonly observed in the sequence.) In summary, we conclude that the frequency of deletion events was very high in (dC-dG) tracts longer than approximately 50 bp (deletions were always visible by gel analysis) and were very low for blocks which were 32 bp or shorter.

These general rules were supported by our attempts to clone oligomers of the 157- or 187-bp inserts, although the 52-bp segment of (dC-dG) was disrupted by GATC. Both recA- and recBC- host cells were tested for their ability to maintain a uniform plasmid population; no host cells have been found to date which have this property. Thus, E. coli has a powerful selection mechanism against long stretches of (dC-dG) regions.

Furthermore, the recombination and supercoiling behaviors of these plasmids were quite unusual since some plasmids showed a marked tendency to form higher plasmid oligomers (dimers, trimers, tetramers, etc.) depending on the position and size of the (dC-dG) insert. This result is especially interesting because of the recent speculation (36) that (dC-dG) and neighboring (dT)-sequences are hotspots for recombination in human fetal globin genes. Also, plasmids containing (dC-dG) tracts invariably were less supercoiled when isolated from E. coli than control plasmids which lacked the sequence with the capability to adopt a left-handed conformation. The reasons for this behavior are uncertain. (dC-dG) tracts may be regarded as inverted repeats such as those found for transposable elements (37). Not only can (dC-dG) regions adopt left-handed conformations (3) but they also have the potential to adopt cruciform structures (38, 39) under appropriate conditions in supercooled molecules. All of these properties may influence the biological instability of (dC-dG) regions in vivo.

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