Isolation and Chromosomal Distribution of Natural Z-DNA-forming Sequences in Halobacterium halobium*

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Jong-myoung Kim† and Shiladitya DasSarma§
From the Department of Microbiology, University of Massachusetts, Amherst, Massachusetts 01003

Conditions favoring left-handed Z-DNA such as high salinity (> 4 M), high negative DNA supercoiling, and GC-rich DNA [statistically favoring d(CG)$_n$ repeat sequences], are all found in the extremely halophilic archaean (archaeabacterium) Halobacterium halobium. In order to identify and study Z-DNA regions of the H. halobium genome, an affinity chromatography method with high Z-DNA selection efficiency was developed. Supercoiled plasmids were incubated with a Z-DNA-specific antibody (Z22) and passed over a protein A-agarose column, and the bound plasmids were eluted using an ethidium bromide gradient. In control experiments using mixtures of pUC12 (Z-negative) and a d(CG)$_n$-containing (Z-positive) pUC12 derivative, up to 4,000-fold enrichment of the Z-DNA-containing plasmid was demonstrated per cycle of the Z-DNA selection procedure. The selection efficiency was determined by transformation of Escherichia coli DH5a with eluted plasmids and blue-white screening on X-gal plates. Twenty recombinant plasmids containing Z-DNA-forming sequences of H. halobium were isolated from a genomic library using affinity chromatography. Z-DNA-forming sequences in selected plasmids were identified by bandshift and antibody footprinting assays using Z22 monoclonal antibody. Alternating purine-pyrimidine sequences ranging from 8 base pairs (bp) to 13 bp with at least a 6-bp alternating d(GC) stretch were found in the Z22 antibody binding regions of isolated plasmids. The distribution of Z-DNA-forming sequences in the Halobacterium salinarum GRB chromosome was analyzed by dot-blot hybridization of an ordered cosmid library using the cloned H. halobium Z-DNA segments as probe. Among the 11 Z-DNA segments tested, five were found to be clustered in a 100-kilobase pair region of the genome, whereas six others were distributed throughout the rest of the genome.

DNA is a flexible and dynamic molecule which can adopt a variety of conformations (1). Under certain conditions, some DNA sequences can change from the most prevalent right-handed B-DNA conformation and adopt unusual DNA conformations, such as left-handed Z-DNA (2), as well as triplex (3, 4), tetraplex (5), and cruciform structures (6). With increasing salt concentration, inversion of the circular dichroism spectrum of poly-d(CG) nucleotides was observed (7), suggesting the occurrence of an unusual DNA form. However, detailed x-ray crystallographic analysis of d(CG) hexamer was required to demonstrate the formation of left-handed Z-DNA (2). Spectroscopic, chemical, and enzymatic analyses have subsequently been used to study the conditions required for Z-DNA formation in vitro (2, 8, 9). In our previous study (10), the factors required for Z-DNA formation were systematically examined by Z-DNA conformational analysis of a series of short alternating d(CG) sequences cloned in plasmids. The results showed that d(CG)$_n$ sequences are in a dynamic equilibrium between B- and Z-DNA forms, while longer alternating d(CG) sequences are predominantly in the Z-form. DNA supercoiling was essential for Z-DNA formation and higher DNA supercoiling favored Z-DNA formation in shorter d(CG) repeat sequences. Surprisingly, salt concentrations as high as 4 M NaCl did not promote Z-DNA formation in these shorter alternating d(CG) sequences to a measurable extent.

Naturally occurring alternating purine-pyrimidine sequences adopting Z-DNA conformation have been found previously in many organisms including prokaryotic and eukaryotic microorganisms, and plants and mammalian cells (2, 9, 11–16). The presence of Z-DNA-forming sequences in putative regulatory regions near the 5′-ends of genes (15) and the inhibition of transcription by RNA polymerase in Z-DNA regions (17) suggested a regulatory role for Z-DNA in gene expression. The finding of greater Z-DNA conformation within transcriptionally active regions of chromosomes (18) was also consistent with a role for Z-DNA in transcription. Involvement of Z-DNA in genetic recombination (19, 20) was supported by the finding of high frequency recombination for plasmids containing Z-DNA, preferential binding of recombinational proteins such as RecA to Z-DNA, and finding of potential Z-DNA-forming d(GT)$_n$ sequences in recombinational hot spots in eukaryotic cells. Other functions for Z-DNA, such as a structural role in nucleosome formation (21) and as the recognition site for DNA topoisomerase II (22), have also been suggested, and several Z-DNA-binding proteins have been isolated (23–25). However, due to the dynamic nature of genetic processes and the transient nature of left-handed Z-DNA conformation, the exact roles of Z-DNA in vivo are still controversial.

To gain deeper insight into the functional role of Z-DNA conformation in vivo, we explored the characteristics of a diversity of organisms and identified H. halobium as an interesting organism for the analysis of left-handed DNA conformation. H. halobium occupies an unusual evolutionary and physiological position, as a member of a group of obligately halophilic archaea (archaebacteria) requiring at least 3 M NaCl for growth. H. halobium contains high (>4 M) salt concentration (26), high (σ = 0.7) negative DNA supercoiling (10, 27), and high (~67%) GC content (28), all of which are known to favor

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† To whom correspondence should be addressed. Tel.: 413-545-2581 or 413-545-1936; Fax: 413-545-1578; E-mail: sds@rna.micro.umass.edu.
Z-DNA formation. We reasoned that Z-DNA is likely to be highly prevalent in the H. halobium genome, and isolation and characterization of these sequences may provide valuable information about the function of Z-DNA sequences generally.

In order to isolate Z-DNA-forming regions of the H. halobium genome, we developed an improved method for affinity chromatography using a Z-DNA-specific monoclonal antibody column (29–31). Using a plasmid series containing d(CG) sequences, the procedure was shown to be highly selective and was used to isolate recombinant plasmids with Z-DNA-forming sequences from an H. halobium genomic library. We found that although salt has no measurable effect on Z-DNA formation in short alternating d(CG) sequences, a large number of Z-DNA-forming sequences do exist in the H. halobium genome.

**EXPERIMENTAL PROCEDURES**

**Materials—**Restriction endonucleases, large fragment of Escherichia coli DNA polymerase I, T4 DNA ligase, S1 nuclease, and T4 polynucleotide kinase were purchased from Life Technologies, Inc. and New England Biolabs. Exonuclease III was purchased from Stratagene, and DNA polymerase I, Klenow fragment of DNA polymerase I, and T7 DNA polymerase were purchased from Promega. Glutaraldehyde, OsO4, and other chemicals were purchased from Sigma. Radiolabeled nucleotides, 9,2-D-deoxyadenosine 5'-32P]triphosphate (3,000 Ci/mmol, 10 mCi/ml), and adenosine 5'-32P]triphosphate (5,000 Ci/mmol, 10 mCi/ml), were purchased from Amersham Corp. Monoclonal antibodies were obtained from Dr. B. D. Stollar (Tufts University, Boston, MA) (31) and DNA dot blots containing Halobacterium salinarum GRB cosmid library DNA (32) were kindly provided by Dr. R. L. Charlebois (University of Ottawa, Ontario, Canada).

**Molecular Biology Methods—**Standard methods of molecular biology including isolation of plasmids by the alkaline-SDS procedure, agarose and polyacrylamide gel electrophoresis, CaCl2-mediated transformation of E. coli, labeling of DNA, etc. were carried out as described (33). DNA sequencing was carried out by the dideoxy chain termination method of Sanger (34) on double-stranded plasmid template using the Sequenase version 2.0 sequencing kit. Southern and dot-blot hybridizations were carried out under stringent conditions as described (33).

Isolation of Z-DNA-containing Plasmids—Z-DNA-containing plasmids were treated with Z22 antibody (31) and isolated by affinity chromatography using protein A-agarose. Protein A affinity chromatography was prepared by packing 0.4 ml of protein A-agarose in a chromatography column. The column was equilibrated with 10 bed volumes of binding buffer (10 ml Tris-HCl (pH 8.75) and 0.1 M NaCl) before application of DNA.

For control experiments, 10 μg mixtures of pUC12 and pJKCG (n = 2, 4, and 5) (constructions described in Ref. 10) were incubated with 0.1 μg of Z22 antibodies in 100 μl of binding buffer at 37 °C for 1 h. The mixture was passed through a protein A-agarose column prepared as above at a flow rate of 0.4 ml/min. The column was washed with 20 column volumes of binding buffer to remove nonspecifically bound DNA. The bound DNA was consecutively eluted with 2 column volumes of binding buffer with increasing ethidium bromide concentration (0.01, 0.5, 5, and 50 μg/ml). Each fraction of column volume was extracted twice with phenol and once with chloroform. The DNA was precipitated with ethanol, bound DNA. The bound DNA in the column was eluted using 0.1 ml of binding buffer at 37 °C overnight.

For selection of Z-DNA-containing plasmids, an H. halobium genomic library, constructed by inserting partial Sau3AI fragments into the BamHI site of pUC12, was amplified (35). The affinity chromatography procedure was carried out as above using 10 μg of library DNA instead of the mixture of pUC12 and pJKCG plasmids. The column fraction corresponding to the one which showed the most enriched pJKCG plasmid in the control experiment, was collected, and the DNA was ethanol-precipitated as described above. The DNA was transformed into E. coli DH5α for plasmid amplification. The selected plasmid population was subjected to three additional rounds of affinity selection and amplification using the same procedure.

Z-DNA Conformational Analysis—Z-DNA conformation in pJKCG and affinity-selected plasmids was first examined by the ZIBS’ (Z-DNA-immuno-bandshift) assay using Z22 monoclonal antibody cross-linking as described (10). Fine mapping of Z-DNA conformation on pJKZ22 was carried out either by S1 nuclease and chemical modification assay (8, 10) or by a Z-DNA antibody footprinting assay (10). For antibody footprinting assay, plasmid DNA was cross-linked with Z22 antibody and the DNA-antibody complex was incubated in 10 μl of reaction buffer containing 50 mM NaCl, 10 mM Tris-HCl (pH 7.9), 10 mM MgCl2, 1 mM dithiothreitol, and 5 units of PvuII. After 3 h of incubation at 37 °C, 10 μl of the above buffer containing 0.5 units of exonuclease III was added to the reaction mixture and further incubated at 37 °C for 30 min. The reaction mixture was passed through a Sephadex G-10 spin column (33) and dried under vacuum. The DNA was dissolved in 16 μl of water and denatured by the addition of 4 μl of 2 n NaOH. After a 10-min incubation at room temperature, the DNA was precipitated by addition of 20 μl of 0.9 M sodium acetate and 120 μl of ethanol. The DNA was collected by centrifugation and then phosphorylated by addition of 5 μl of 2 × Klenow buffer containing 2.5 pmol of [γ-32P]ATP and 2 μl of T4 polynucleotide kinase. The reaction mixture was subjected to electrophoresis on a 6% denaturing polyacrylamide gel.

For S1 nuclease assays, 1 μg of plasmid DNAs were incubated with 0.1 unit of S1 nuclease at 37 °C for 10 min in 20 μl of 50 mM sodium acetate (pH 4.6), 50 mM NaCl, and 1 mM zinc acetate. The reaction mixtures were extracted twice with phenol and once with chloroform, and the DNA was precipitated with ethanol as above. The site of cleavage was mapped by primer extension using [32P]-labeled forward or reverse sequencing primers and dideoxynucleotide sequencing mixture and electrophoresis on 8% polyacrylamide, 7 M urea gels (10). For OsO4 modification assays, plasmid DNAs were incubated in a 50-μl solution containing 25 nm Tris-HCl (pH 7.5), 0.1 M NaCl, 2 mM EDTA, 2 mM OsO4, and 2 mM bipyridimidine at 37 °C for 10 min. The reaction mixtures were passed through a Sephadex G-50 spin column, and the DNA was precipitated with ethanol. OsO4-sensitive sites were mapped by primer extension analysis as described above.

**RESULTS**

Z-DNA Affinity Chromatography Using Z22 Antibody and Protein A-Agarose Column—In order to isolate naturally occurring DNA sequences adopting Z-DNA conformation, a highly selective affinity chromatography method was developed. The method involved incubation of plasmid DNA with Z22 monoclonal antibody specific for Z-DNA, followed by chromatography through a protein A-agarose column. The Z-DNA-containing plasmids that were complexed to antibody were retained on the column via protein A-antibody bridges, whereas other plasmids lacking Z-DNA were eluted. Bound plasmids were eluted by increasing concentrations of ethidium bromide, which unwinds the DNA by intercalation and converts Z-DNA segments into B-DNA conformation. This led to loss of antibody binding and elution of Z-positive plasmids from the column.

To determine the efficiency of the affinity chromatography to select Z-positive plasmids, control experiments using mixtures of pUC12 and pJKCG (n = 2, 4, and 5) were performed. The total number of transformants. Transformants containing pUC12 produced blue colonies on plates with X-gal, whereas transformants containing pJKCG2, pJKCG4, and
pJKCG5 plasmids produced white colonies, due to frameshifts in the LacZ coding region caused by the d(CG)n inserts. Therefore, enrichment by affinity purification could be easily determined by counting the number of blue and white colonies.

Fig. 1 shows the plasmid elution profile using the buffer containing ethidium bromide. Not shown is that more than 90% of plasmids applied to the column were either not bound or washed off the column by buffer lacking ethidium bromide. Plasmids were quantitatively released with 0.5 µg/ml and 5 µg/ml ethidium bromide with the highest enrichment of Z-positive plasmids observed at the higher concentration. Better enrichment was observed for pJKCG5 than for pJKCG4, as would be expected from our earlier finding that the insert in 90% of the former and 20% of the latter are in the Z-form (10). Using 10:1 mixtures of pUC12:pJKCG4 or pJKCG5, about 10-fold enrichment of pJKCG4 and 1,000-fold enrichment of pJKCG5 were observed in the peak fractions. A maximum of 4,000-fold enrichment was demonstrated using a 100:1 mixture of pUC12 and pJKCG5 (data not shown).

Isolation of Natural Sequences Adopting Z-DNA from an H. halobium Genomic Library—After determining the high selectivity of our affinity chromatography procedure in control experiments using mixtures of pUC12 and pJKCG plasmids, we used plasmid DNA amplified from an H. halobium genomic library to isolate natural sequences capable of adopting Z-DNA conformation. The genomic library had previously been constructed by inserting partial Sau3AI fragments into the BamHI site of pUC12 (35). The genomic plasmid library was incubated with Z22 antibody, and the mixture was passed over a protein A-agarose column. After washing the column with an excess amount of binding buffer, Z-DNA-antibody complex bound to protein A-agarose was eluted with buffer containing ethidium bromide. The fractions corresponding to the most enriched fractions were collected and transformed into E. coli DH5α for amplification. Plasmid DNA was isolated from a culture of the total mixture of transformed cells. The plasmid population enriched for Z-positive plasmids was subjected to a second, third, and fourth cycle of Z-DNA selection by affinity chromatography to obtain plasmid mixtures increasingly enriched for Z-DNA.

Plasmids isolated from each cycle of Z-DNA selection procedure were subjected to agarose gel electrophoresis to determine if specific plasmids were selected from the genomic library (Fig. 2). Library DNA before selection for Z-positive plasmids was highly complex and appeared as a smear upon electrophoresis (lane 2), whereas distinct bands appeared after successive passages through the affinity column (lanes 3–6). This confirmed that the procedure is selective for Z-DNA-containing plasmids with greater purity in successive cycles of chromatography. After four cycles of Z-DNA enrichment and amplification, plasmids were prepared in large scale and electrophoresed on a preparative agarose gel. After staining the gel with ethidium bromide, 20 distinct bands were observed. Each was purified by electroelution and transformed into E. coli DH5α for DNA amplification. Plasmid DNAs obtained from gel purified Z-positive bands were designated as pJKZn, where n refers to the identity of individual plasmids. Some bands yielded multiple Z-positive plasmids.

Analysis of Z-DNA Conformation in Recombinant Plasmids—The presence and approximate location of Z-DNA 
Hin incubated with Z22 antibody, digested with BssHII and labeled with [α-32P]dATP by Klenow fragment of E. coli DNA polymerase I. The bands containing the Z-DNA segment were shifted-up due to antibody cross-linking and the band intensity decrease was used as the criterion for Z-DNA formation. Each isolated plasmid was subjected to the ZIBS assay and the results for several, pJKZ73, 81, 162, 173, 182, 183, 191, 192, and 201, are shown in Fig. 3. The intensity of one Hinfl restriction fragment in each plasmid decreased upon antibody cross-linking (even-numbered lanes) compared to the same fragment intensity without antibody cross-linking (odd-numbered lanes). The occurrence of antibody binding reflected by a clearly shifted band was detected in 20 distinct recombinant plasmids, although some differences in the extent of bandshift probably reflecting the strength of antibody binding was observed. All of the Z-DNA-containing plasmids isolated are listed in Table I.

Further analysis of Z-DNA conformation in recombinant plasmids isolated by affinity chromatography was carried out by digestion with BssHII. Since BssHII recognizes the sequence G'CGCCG, the occurrence of short (dG-dC) repeats, the most likely Z-DNA-forming sequence, could be tested. The results of restriction digestion and agarose gel electrophoresis (data not shown, Ref. 36) indicate that all isolated plasmids were cleaved by BssHII. Since there is no BssHII recognition sequence in the pUC12 vector portion, cleavage by BssHII indicates that all inserts contain at least one (dG-dC) repeat sequence (Table I). Several plasmids, such as pJKZ32, pJKZ51, and pJKZ63, contained many BssHII cleavage sites. These results indicate that the Z-positive plasmids may contain multiple Z-DNA-forming (dG-dC) repeat sequences.

Since d(CG) repeats have high potential to form Z-DNA, it was of interest to analyze whether BssHII recognition sites indeed correlate to Z22 antibody binding and Z-DNA formation. For all plasmids tested, antibody-binding Hinfl restriction fragments were cleaved by BssHII (data not shown, Ref. 36). These results suggested that the Z-DNA-forming sequences isolated may contain alternating purine-pyrimidine sequences with at least one (dG-dC) repeat.

DNA Sequence Analysis of Z-DNA-forming Regions—In order to obtain more information about DNA sequences adopting Z-DNA conformation, limited DNA sequence analysis of several pJKZn plasmids was carried out. DNA sequences of plasmids with smaller inserts were first determined using the universal sequencing primer. pJKZ22 contains the smallest insert among isolated plasmids, and DNA sequence analysis established the sequence of the entire 42-bp insert (Fig. 4A). A potential Z-DNA-forming sequence composed of d(CG)3.5 and (dG-dC)4 connected by 2 bp out of alternation was found. DNA sequence information of pJKZ11 and pJKZ22 was also obtained. pJKZ11 contains an 11-bp alternating purine-pyrimidine sequence including a d(CG)4 repeat (Fig. 4B), and pJKZ22 contains a 13-bp alternating purine-pyrimidine sequence including a (dG-dC)4 repeat sequence (Fig. 4C). DNA sequence of the Z-DNA-forming region of pJKZ32 was obtained after subcloning the antibody-binding Hinfl fragment of pJKZ32 into pUC12. It contains a potential Z-DNA-forming 11-bp alternating purine-pyrimidine sequence including a (dG-dC)4 repeat (Fig. 4D).

Cross-hybridization of pJKZn plasmids using pJKZ32, pJKZ51, and pJKZ63 as probes showed strong homology between pJKZ12 and pJKZ63 (data not shown). Considering the small size of pJKZ12 as compared to pJKZ63 (Table I), it was possible that the cloned fragment in pJKZ12 could be a subfragment of the insert in pJKZ63. For detailed analysis of pJKZ63 and pJKZ12, three TaqI restriction fragments (304, 103, and 79 bp) of pJKZ12 and 1.05-kb AflII fragments of pJKZ63 were subcloned into the Smal site of pUC12. Among the three TaqI fragments of pJKZ12, Z-DNA formation was found in the 260-bp TaqI fragment but not the other TaqI fragments (data not shown, Ref. 36). DNA sequence analysis was carried out on the subcloned TaqI fragments of pJKZ12 and 1.05-kb AflII fragment of pJKZ63 using the universal sequencing primer, which confirmed that the two plasmids contain the same cloned region. The complete DNA sequence of the H. halobium insert in pJKZ12 was obtained (Fig. 4E), and the potential Z-DNA-forming alternating purine-pyrimidine sequence was found in the 260-bp TaqI fragment of pJKZ12. Our results show that every plasmid isolated by Z-DNA affinity chromatography and characterized by DNA sequence analysis contains at least an 8-bp stretch of alternating purine-pyrimidine sequence, including a short d(CG)n repeat sequence.

Fine Mapping of Z-DNA-forming Sequence—To confirm the results of DNA sequencing and ZIBS assays, which provide suggestive and approximate information regarding Z-DNA-forming sequences, antibody footprinting assay, S1 nuclease sensitivity, and OsO4 modification assays were used for fine mapping of Z-DNA-forming sequences. These assays were carried out on pJKZ22, which contains d(CG)3.5 and (dG-dC)4 repeat sequences separated by 2 nucleotides (Fig. 4A). An antibody footprinting experiment was carried out to probe the site of Z22 antibody binding and Z-DNA formation in pJKZ22 (Fig. 5). After cross-linking the DNA-antibody conjugate, it was digested with PvuII restriction endonuclease, which cleaves at sites flanking the Z-DNA region. Exonuclease III digestion was carried out on the antibody cross-linked PvuII fragment from its 3'-ends to the antibody binding region. Protected strands were 5'-end-labeled using T4 polynucleotide kinase and [γ-32P]ATP and resolved by denaturing polyacrylamide-urea gel electrophoresis. The region of Z-DNA-forming sequences protected by Z22 antibody binding was inferred from the distance from the PvuII cleavage sites to the limit of exonuclease III digestion. pJKCG6, known to adopt Z-DNA conformation (10), was included as a positive control for Z-DNA formation using this assay (lanes 1 and 2). The distances between PvuII sites...
and d(CG)6 junctions were 124 nucleotides at one end and 191 nucleotides at the other end. Considering the length of the Z-DNA-forming d(CG)6 stretch, fragments of the above sizes plus 12 nucleotides (136 and 203 nucleotides) were expected to be seen. Protected fragments of about 135 and 204 nucleotides were observed for pJKCG6, consistent with the prediction, and confirming the Z-DNA conformation of the d(CG)6 sequence in this plasmid (Fig. 5, lanes 1 and 2). This result also confirmed the potential of this Z-DNA-antibody footprinting assay to be used as a Z-DNA detection method. In plasmid pJKZ22, protected fragments of about 155 and 216 nucleotides were observed, indicating that the imperfect 17-bp alternating d(CG) repeat sequence (d(CG)3.5 and d(CG)4 connected by 2 bp out of alternation) within the 42-bp insert was bound and protected by antibody due to Z-DNA formation. An extra band of unknown significance was also observed at the lower exonuclease concentration (Fig. 5, lanes 1 and 3).

Z-DNA formation in pJKZ22 was also confirmed by S1 nuclease and OsO4 sensitivity assays. After partial S1 nuclease cleavage or OsO4 treatment, the sensitive sites were identified by primer extension and electrophoresis next to a DNA sequencing ladder (Fig. 6). In control experiments, hypersensitivity was observed at both ends of the d(CG) junction in pJKCG6 (data not shown). For pJKZ22, OsO4 and S1 nuclease-sensitive sites were found flanking the imperfect 17-bp alternating d(CG) sequence containing d(CG)3.5 and d(CG)4 connected by 2 bp out of alternation. The data are provided for only the end near the d(CG)3.5 region following the GATC sequence.

| Z-DNA regions | Size of cloned region (kb) | Number of BssHII sites | Antibody binding | Cosmids hybridized
|---------------|------------------------|----------------------|------------------|-------------------|
| Z11           | 0.5                    | 2                    | + + +            | G18H11, G29G6     |
| Z12           | 0.55                   | 1                    | + + +            | G6A12, G20A8      |
| Z21           | 0.6                    | 2                    | + + +            | G12E9             |
| Z22           | 0.04                   | 2                    | + + +            | G19E12            |
| Z32           | 4.5                    | 14                   | + + +            |                  |
| Z33           | 1.0                    | 2                    | ND               |                  |
| Z43           | 1.2                    | 2                    | + + +            | G7D12             |
| Z51           | 4.5                    | 15                   | + + +            | G14B2             |
| Z52           | 1.25                   | 4                    | + + +            | G5H7, G14F11, G14B2 |
| Z53           | 2.7                    | 5                    | + + +            | G6A12, G9G1       |
| Z61           | 2.8                    | 6                    | + + +            |                  |
| Z62           | 4.5                    | 7                    | + + +            |                  |
| Z63           | 4.0                    | 8                    | + + +            |                  |
| Z64           | 1.3                    | 2                    | + + +            |                  |
| Z102          | 2.2                    | 3                    | + + +            |                  |
| Z103          | 3.5                    | 4                    | + + +            |                  |
| Z104          | 1.2                    | 2                    | + + +            |                  |
| Z105          | 1.5                    | 4                    | + + +            |                  |
| Z106          | 3.5                    | 6                    | + + +            |                  |
| Z107          | 4.5                    | 7                    | + + +            |                  |
| Z108          | 4.0                    | 8                    | + + +            |                  |
| Z109          | 1.3                    | 2                    | + + +            |                  |
| Z110          | 2.2                    | 3                    | + + +            |                  |
| Z111          | 3.5                    | 4                    | + + +            |                  |
| Z112          | 1.2                    | 2                    | + + +            |                  |
| Z113          | 1.5                    | 4                    | + + +            |                  |
| Z114          | 3.5                    | 6                    | + + +            |                  |
| Z115          | 4.5                    | 7                    | + + +            |                  |
| Z116          | 4.0                    | 8                    | + + +            |                  |
| Z117          | 1.3                    | 2                    | + + +            |                  |
| Z118          | 2.2                    | 3                    | + + +            |                  |
| Z119          | 3.5                    | 4                    | + + +            |                  |
| Z120          | 1.2                    | 2                    | + + +            |                  |
| Z121          | 1.5                    | 4                    | + + +            |                  |
| Z122          | 3.5                    | 6                    | + + +            |                  |

**Table I**

Characteristics of cloned Z-DNA regions of *H. halobium*

| Z-DNA regions | Size of cloned region (kb) | Number of BssHII sites | Antibody binding | Cosmids hybridized |
|---------------|------------------------|----------------------|------------------|-------------------|
| Z11           | 0.5                    | 2                    | + + +            | G18H11, G29G6     |
| Z12           | 0.55                   | 1                    | + + +            | G6A12, G20A8      |
| Z21           | 0.6                    | 2                    | + + +            | G12E9             |
| Z22           | 0.04                   | 2                    | + + +            | G19E12            |
| Z32           | 4.5                    | 14                   | + + +            |                  |
| Z33           | 1.0                    | 2                    | ND               |                  |
| Z43           | 1.2                    | 2                    | + + +            | G7D12             |
| Z51           | 4.5                    | 15                   | + + +            | G14B2             |
| Z52           | 1.25                   | 4                    | + + +            | G5H7, G14F11, G14B2 |
| Z53           | 2.7                    | 5                    | + + +            | G6A12, G9G1       |
| Z61           | 2.8                    | 6                    | + + +            |                  |
| Z62           | 4.5                    | 7                    | + + +            |                  |
| Z63           | 4.0                    | 8                    | + + +            |                  |
| Z64           | 1.3                    | 2                    | + + +            |                  |
| Z102          | 2.2                    | 3                    | + + +            |                  |
| Z103          | 3.5                    | 4                    | + + +            |                  |
| Z104          | 1.2                    | 2                    | + + +            |                  |
| Z105          | 1.5                    | 4                    | + + +            |                  |
| Z106          | 3.5                    | 6                    | + + +            |                  |
| Z107          | 4.5                    | 7                    | + + +            |                  |
| Z108          | 4.0                    | 8                    | + + +            |                  |
| Z109          | 1.3                    | 2                    | + + +            |                  |
| Z110          | 2.2                    | 3                    | + + +            |                  |
| Z111          | 3.5                    | 4                    | + + +            |                  |
| Z112          | 1.2                    | 2                    | + + +            |                  |
| Z113          | 1.5                    | 4                    | + + +            |                  |
| Z114          | 3.5                    | 6                    | + + +            |                  |
| Z115          | 4.5                    | 7                    | + + +            |                  |
| Z116          | 4.0                    | 8                    | + + +            |                  |
| Z117          | 1.3                    | 2                    | + + +            |                  |
| Z118          | 2.2                    | 3                    | + + +            |                  |
| Z119          | 3.5                    | 4                    | + + +            |                  |
| Z120          | 1.2                    | 2                    | + + +            |                  |
| Z121          | 1.5                    | 4                    | + + +            |                  |
| Z122          | 3.5                    | 6                    | + + +            |                  |

*a*, moderate antibody binding; **+, stronger antibody binding; ++++, strongest antibody binding; ND, not determined.

b Cosmids dot-blot were obtained from Dr. R. Charlebois (32).

**Fig. 5. Detection of the Z-DNA-forming region in plasmid KZ22 by exonuclease digestion.** Locations of Z22 antibody binding in plasmid KCG6 and plasmid KZ22 were determined by mapping the region of PvuII fragments protected from exonuclease III digestion. Lanes 1 and 2 contain plasmid KCG6, and lanes 3 and 4 contain plasmid KZ22 treated with 0.5 and 1.5 units of exonuclease III, respectively. Lanes labeled G, A, T, and C contain sequencing reactions for size markers using plasmid KZ22 template. Sizes are indicated on the right in nucleotides.

**Fig. 4. Nucleotide sequence of Z-DNA-containing fragments of plasmid KZ22.** Complete DNA sequence of plasmid KZ22 (A) and plasmid KZ22 (E) inserts and antibody-binding Hinfl restriction fragment (D) of plasmid KZ22 are shown. Partial DNA sequence information of plasmid KZ11 (B) and plasmid KZ21 (C) are also shown. Potential Z-DNA-forming alternating purine-pyrimidine sequences are underlined.
The sensitivity at the ends of the alternating d(CG) sequences indicates that these sequences are in Z-DNA conformation. Mapping of pJKZ21 and pJKZ32 using S1 nuclease and OsO4 (data not shown) also showed the existence of B-Z junctions flanking the alternating purine-pyrimidine sequences in these plasmids (see Fig. 4, C and D).

Distribution of Z-DNA-forming Sequences in the Genome—The location of Z-DNA-containing segments were analyzed by dot-blot hybridization of cosmids representing the genome of H. salinarum GRB (32), which has a similar chromosomal map to H. halobium NRC-1. Membranes containing cosmids DNA were hybridized with 32P-labeled restriction fragments encompassing Z-DNA regions. Z-positive plasmids digested with HinfI were electrophoresed in agarose gels, and the antibody binding fragments identified by ZIBS assay were purified by electrophoresis. The purified Z-DNA-containing fragments were labeled by nick translation in the presence of [α-32P]dATP and used as a probe in hybridization (data not shown). Except for a few cloned Z-DNA-containing restriction fragments that showed multiple hybridizations, most of the fragments hybridized to a single or two overlapping cosmids (Table I). The location of the Z-DNA region in the genome was deduced from the known cosmid map information of H. salinarum GRB genome and is represented in Fig. 7. Six Z-DNA segments including those cloned in pJKZ12, pJKZ53, pJKZ63, pJKZ183, pJKZ192, and pJKZ201 were found clustered in the genomic region between 200 and 300 kb, whereas six others were distributed throughout the rest of the genome.

DISCUSSION

In order to gain insight into the functional role of Z-DNA conformation in vivo, we identified H. halobium as an especially interesting organism. H. halobium contains high (>4 M) salt concentration, high (ρ = 0.7) negative DNA supercoiling, and high (67%) GC content, all of which are known to favor Z-DNA conformation. Initially, we carried out in vitro studies to carefully define the parameters important for Z-DNA formation (10). Next, we developed an improved method for selection of Z-DNA-forming regions of the H. halobium genome using a Z-DNA-specific monoclonal antibody. Using control plasmids with or without d(CG)n sequences, the procedure was shown to be highly selective and was used to isolate recombinant plasmids with Z-DNA-forming sequences from an H. halobium genomic library. Our studies established that many sequences capable of forming Z-DNA do exist and are distributed throughout the H. halobium chromosome. This finding was unexpected given the results from earlier studies, which showed that salt has no measurable effect on Z-DNA formation in the shortest alternating dC-dG sequences in plasmids (10, 37).

Our approach required the development of an improved method for isolating sequences capable of adopting Z-DNA conformation. DNA sequences that adopt Z-DNA conformation in supercoiled plasmids were bound to the Z-DNA-specific Z22 monoclonal antibodies. A very low concentration of Z22 antibody was used to minimize the likelihood of antibody-induced
Z-DNA formation. Plasmid-antibody complexes that were retained on a protein A column were subsequently eluted by increasing concentrations of ethidium bromide. In a previous report, Z-DNA antibody-DNA complexes were bound to nitrocellulose, and a one step elution with a high concentration of ethidium bromide was used to release the bound DNA (30). However, in control experiments, we found that most of the DNA eluted with a single step of ethidium bromide is not highly enriched for plasmids known to contain Z-DNA segments. Therefore, a stepwise increase in ethidium bromide concentration was used in this study to obtain greater selectivity for Z-DNA-containing sequences. Maximum enrichment (up to 4,000-fold) was obtained for Z-positive molecules from a 1:100 mixture of pUC12 and pJKG5.

Our affinity purification method permitted us to isolate 20 recombinant plasmids containing natural H. halobium sequences capable of adopting Z-DNA conformation. Multiple cycles of affinity purification was used to increase selectivity for Z-positive plasmids, which was apparent by agarose gel electrophoresis. However, in the fourth round of selection, a few plasmids were lost (compare lanes 5 and 6 in Fig. 2). This result may have been due to saturation of the column with Z-positive molecules or preferential binding of plasmids with greater propensity to form Z-DNA. The presence of antibody binding fragments in almost all selected plasmids confirmed the high Z-DNA selection efficiency of the Z-DNA isolation method. The specificity of the procedure was also confirmed by the repeated cloning of identical regions of the genome adopting Z-DNA. For example, Southern hybridization experiments showed that the pJKZ12 insert is a subfragment of the insert in pJKZ63. Moreover, all isolated sequences contained one or more BssHII recognition sites in the Z22-binding restriction fragments, indicating the presence of (dG-dC)3 or longer sequences known to favor left-handed structure.

We also determined the nucleotide sequences corresponding to the Z-positive regions of pJKZ11, pJKZ12, pJKZ21, pJKZ22, and pJKZ32. Alternating purine and pyrimidine sequences were found in all selected plasmids ranging from 8 to 13 bp of (dG-dC)6 sequence. Based on our previous results using model plasmids (10), alternating purine-pyrimidine sequence of these lengths are in dynamic equilibrium between B- and Z-DNA conformations in supercoiled plasmids. Therefore, it is likely that these DNA regions are undergoing conformational transitions in response to changes in intracellular conditions such as local DNA supercoiling or protein binding.

The possible functions of the isolated H. halobium sequences capable of adopting Z-DNA conformation are still unclear. Preliminary experiments were carried out to assay for transcripts encoded by restriction fragments bearing Z-DNA segments (data not shown). Interestingly, a transcript encoded by the cloned insert in pJKZ32 was detected and was found at a higher level in the purple membrane-overproducing H. halobium strain S9 than in NRC-1. Since S9 is a regulatory mutant, this result is consistent with a possible regulatory role for the Z-positive conformation. In a separate report (27), we have also identified an 11-bp alternating purine-pyrimidine sequence centered 23 bp upstream of the opd gene transcription start point, which is capable of adopting a non-B-DNA structure not unlike Z-DNA (Fig. 7). In this case, the data are consistent with a DNA supercoiling-dependent B-Z switch regulating opd transcription.

The location and distribution of Z-DNA segments in the H. halobium genome were examined by hybridization to cosmids representing the genome of the closely related H. salinarum GRB strain. Eleven regions of Z-DNA-containing sequences were mapped to specific sites in the genome. Five Z-DNA segments were located in the region between 200 and 300 kb, which was inverted in H. halobium NRC-1 and S9 (38) relative to H. salinarum GRB, whereas six other Z-DNA segments were scattered throughout the remainder of the chromosome. We also searched GenBank data base for homologous genes and encoded proteins to the Z-DNA segments sequenced using the FASTA and TFASTA programs (39). However, no homologous genes or proteins could be identified.

It is known that the H. halobium genome is unstable due to frequent DNA rearrangements (in contrast to the H. salinarum GRB genome, which is more stable) (40). To study the possible involvement of Z-DNA in genomic instability, we compared the sizes of Z-DNA-containing restriction fragments of several related Halobacterium species and strains by Southern hybridization. If Z-DNA was involved in genomic rearrangements, the sizes of Z-DNA-containing fragments would likely have been altered in the different strains. However, very few differences were observed (data not shown, 36), suggesting that Z-DNA may not be involved in extensive DNA rearrangement.

We have found many sequences in the H. halobium genome capable of adopting left-handed Z-DNA conformation. The sequences are generally short alternating purine-pyrimidine repeats, rich in G and C nucleotides, and show the ability to adopt Z-DNA conformation when cloned in plasmids of E. coli. In H. halobium, the higher DNA supercoiling density, if not the higher salt concentration (10, 37), would help to stabilize left-handed DNA structure. These findings indicate that a wide variety of prokaryotic microorganisms, especially those with GC-rich genomes, probably contain multiple regions capable of adopting Z-DNA conformation. The consequence of Z-DNA structure in these regions on genetic phenomena awaits further study.

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