Intrastrand Self-complementary Sequences in *Bacillus subtilis* DNA

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Intrastrand self-complementary sequences have been isolated from the DNA of *Bacillus subtilis* by hydroxyapatite (HA) chromatography following thermal renaturation of strands separated by chromatography on methylated albumin–kieselguhr (MAK). The intrastrand structures derived from the MAK H strand (HA HII) were biologically active showing transforming activity for a wide variety of markers, as well as hybridization to both pulse-labelled and ribosomal RNA. Removal of regions of single-strand DNA with S1 nuclease did not significantly alter the biological activity of the self-annealed molecules. The overall efficiency of transformation and hybridization of the intrastrand self-annealing DNA was low suggesting that many sequences in the population are neither active in transformation to prototrophy nor transcribed into RNA.

INTRODUCTION

The presence of self-complementary sequences is a widespread phenomenon, occurring in the DNA of higher eukaryotes (Wilson & Thomas, 1974), in bacteriophages M13 (Forsheit & Ray, 1970), fd (Schaller *et al.*, 1969) and f1 (Shishido *et al.*, 1969), and in bacterial plasmids (Sharp *et al.*, 1973). Kato *et al.* (1974) have reported that 2 to 8% of the DNA from *Escherichia coli* is capable of intrastrand base pairing. In general, secondary structure in the DNA of prokaryotes is less well-characterized than in the DNAs of other organisms. The purpose of our studies was to characterize the intrastrand secondary structure found in the DNA of *Bacillus subtilis* and to investigate the possible biological implications of such structures.

Rudner & Remeza (1973) have previously reported that intrastrand secondary structure can be detected in *B. subtilis* DNA. Strands of DNA were resolved by methylated albumin–kieselguhr chromatography (Roger *et al.*, 1966; Rudner *et al.*, 1968, 1969) and the molecules which were capable of annealing to form secondary structures were separated from the rest of the DNA by hydroxyapatite chromatography (Bernardi, 1969; Rudner & Remeza, 1973). The rate of formation of these structures was rapid and independent of DNA concentration indicating intrastrand reassociation (Rudner & Remeza, 1973). Genetic markers which are widely scattered throughout the *B. subtilis* genome were transformed by the DNA which contained secondary structure suggesting that the location of such sequences is not unique. The biological nature of the self-annealing DNA has been further characterized using transformation assays and by hybridization of this DNA to mRNA and to purified species of stable RNA. DNA which had been digested with S1 nuclease to remove single-strand regions (Ando, 1966) was also analysed by these methods. We report here that the self-annealing DNA partly comprises sequences which are transcribed into RNA and which are active in DNA-mediated bacterial transformation; however, the DNA which forms intrastrand secondary structures also contains sequences which are biologically silent.

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METHODS

Bacterial strains. Mutant derivatives of *Bacillus subtilis* 168 were used: A26 (*ura-1*), for the preparation of DNA and radioactively labelled RNA; BD-204 (*hisB2 thyA thyB*), for the preparation of radioactively labelled DNA; BD-170 (*thr-5 trpC2*) and Mu8u5u16 (*leu-8 metB5 purA16*), as recipients in transformation assays.

Transformation assays. Competent cells were prepared by a modification of the two-step growth method of Anagnostopoulos & Spizizen (1961). Media I and II were as described by Rudner *et al.* (1967). Cells were either used immediately or concentrated 10-fold by centrifugation, adjusted to 10% (v/v) glycerol and frozen. DNA (0.1 ml) was added to 0.9 ml of competent cells and incubated for 30 min at 37 °C; then pancreatic DNAase (Worthington; 10 µg ml⁻¹ in 0.2 M-MgSO₄) was added. The cultures were diluted in saline and plated on selective media as described by Rudner *et al.* (1967).

Isolation, denaturation and strand separation of DNA. *Bacillus subtilis* DNA was extracted according to a modification (Rudner *et al.*, 1967) of the Marmur (1961) procedure. It was denatured at concentrations of 25 to 40 µg ml⁻¹ in alkali as described by Rudner *et al.* (1968). The strands of *B. subtilis* DNA were separated by methylated albumin-kieselguhr (MAK) chromatography using an intermittent salt gradient as described previously (Rudner *et al.*, 1968, 1969; Rudner & Remeza, 1973).

Hydroxyapatite chromatography of self-annealed DNA. The isolated strands of DNA obtained by MAK chromatography were self-annealed at 68 °C for 2 h at the salt concentration at which the strands were eluted from the MAK column. The DNA was then dialysed against 0.05 M-sodium phosphate buffer (pH 6.7) and applied to hydroxyapatite (HA) columns (1 cm diam. × 1 cm per A₂₆₀ of DNA applied). The hydroxyapatite was prepared as described by Miyazawa & Thomas (1965). The DNA was eluted with 250 to 300 ml of a linear gradient of 0.05 to 0.5 M-sodium phosphate buffer (pH 6.7) at a flow rate of 30 ml h⁻¹.

Digestion with S1 nuclease. S1 nuclease isolated from *Aspergillus oryzae* (Ando, 1966) was purchased from Miles Laboratories. The reaction mixture contained 0.3 M-sodium acetate (pH 4.5), 1 µM-ZnCl₂, 0.1 M-NaCl, 20 µg sheared, denatured calf thymus DNA ml⁻¹, radioactive DNA, and 500 units of enzyme (as determined by Miles Laboratories). The mixture was incubated at 37 °C for 1 h. Samples were then removed and either precipitated with 10% (w/v) trichloroacetic acid and prepared for radioactive counting, or extracted twice with phenol and used in transformation or hybridization assays.

Isolation and fractionation of RNA. Two [³H]uridine-labelled RNA preparations isolated from exponentially growing vegetative cells were used: (i) from cells pulse-labelled for 3 min; (ii) from cells labelled continuously for 3 to 4 generations followed by treatment with actinomycin D. Both RNA preparations were made by Dr Yoshiko Setoguchi according to published procedures (Margulies *et al.*, 1970, 1971). The long-labelled RNA was fractionated into components by MAK chromatography as described by Margulies *et al.* (1970).

Hybridization to RNA. Two methods of DNA-RNA hybridization were used: (i) the liquid technique as described by Nygaard & Hall (1963) and modified by Kennell & Kotoulas (1968); (ii) the filter technique described by Gillespie & Spiegelman (1965). In the liquid method, each reaction mixture contained not less than 2 µg DNA ml⁻¹ and [³H]uridine-labelled RNA in a total volume of 0.05 to 0.10 ml. The hybridization was carried out at 68 °C for 18 h in 6×SSC buffer (SSC buffer is 0.15 M-NaCl/0.015 M-sodium citrate, pH 7.0); it was terminated by chilling on ice and diluting with 10 ml 2×SSC buffer. The hybrids were collected on nitrocellulose filters (B-6, 24 mm; Schleicher & Schuell Co.), washed with 100 ml 2×SSC buffer, treated with RNAase A (20 µg ml⁻¹) and RNAase T1 (20 units ml⁻¹) (both from Worthington) for 1 h at room temperature, washed again with 100 ml 2×SSC buffer, dried and counted in a toluene-based scintillant.

In the filter method, 10 µg ³²P-labelled DNA were slowly added to a nitrocellulose filter which previously had been soaked in 0.1 M-KOH for 30 min and then in 2×SSC buffer for 30 min. The filters containing DNA were washed with 100 ml 2×SSC buffer, dried at room temperature and baked for 2 h at 80 °C *in vacuo*. The dried filters were incubated in scintillation vials with [³H]uridine-labelled RNA in 6×SSC buffer in a total volume of 1.0 ml, at 68 °C for 16 h. The reactions were terminated and filters were washed, treated with RNAases and prepared for radioactive counting as described above.

Sedimentation velocity centrifugation. Sedimentation velocity studies were performed using a Beckman model E Analytical Ultracentrifuge equipped with ultraviolet absorption optics. A 30 mm single sector cell, Kel-F 4° centrepiece and quartz windows were used. A Beckman model RB Analytrol was used to obtain densitometer tracings of the photographic films. Centrifugation was done at 24000 rev. min⁻¹ at 20 °C with DNA at 8 to 30 µg ml⁻¹ in 1×SSC/0.2 M-NaOH. (The native DNA sample was kept at neutral pH.) The sedimentation coefficient was obtained from the relation: $s = (1/w^2) (d \ln x / dr)$ where $d \ln x / dr$ is the slope of the line of the natural logarithm of the distance from the centre of rotation to the boundary versus the time of

sedimentation (Svedberg & Pederson, 1940). The molecular weights of the DNAs were estimated according to Studier (1965):

$$\text{for native DNA } s_{20,w}^0 = 0.0882 M^{0.346} \quad \text{for alkaline DNA } s_{20,w}^0 = 0.0528 M^{0.400}$$

RESULTS

Transforming activity of fractionated DNA

Alkali-denatured *B. subtilis* DNA has been separated into two fractions, designated L and H, by MAK chromatography (Fig. 1a) and the complementarity of these fractions has been well substantiated (Rudner *et al.*, 1968, 1969; Karkas *et al.*, 1968). In addition, a minor component (M) of 'native-like' molecules of DNA containing covalent cross-links (Alberts, 1968) elutes at a lower salt molarity than the L fraction. All of the residual transforming activity of denatured *B. subtilis* DNA is associated with the cross-linked molecules (Rownd *et al.*, 1968). Another distinctly different population of molecules which has transforming

Fig. 1. Transforming activity following thermal renaturation of individual fractions of DNA separated by MAK (a) and HA (b) chromatography.

(a) Alkali-denatured DNA (3.7 mg) was applied in 0.7 M-NaCl in 0.05 M-sodium phosphate buffer (pH 6.8) and eluted with 500 ml of 0.7 to 1.5 M-NaCl. The gradient was interrupted at tube 21 and re-established at tube 26. Recovery was 76.3% with an L/H ratio of 1.15 as determined by A_{260} (—). Individual fractions were heated at 68 °C for 2 h and adjusted to 5 $\mu\text{g ml}^{-1}$. Transformation for *thr-5* was assayed using BD170 recipient cells and 0.5 $\mu\text{g DNA ml}^{-1}$. At 0.5 $\mu\text{g ml}^{-1}$ the activity of native DNA was 2.3×10^5 transformants ml^{-1} . The transforming activity (---) is expressed as a percentage of the original transforming activity.

(b) Fractions 38 to 45 of MAK H DNA were renatured at 68 °C, dialysed against 0.05 M-sodium phosphate buffer (pH 6.7), adsorbed to a 6 cm HA column, and eluted with 250 ml of 0.05 to 0.5 M-sodium phosphate buffer (pH 6.7). The recovery (—) as a percentage of the input was: total, 72.3; HI, 47.0; HII, 23.0. The transforming activity (---) was assayed as in (a).

Table 1. *Effect of S1 nuclease digestion on transforming activity*

Unlabelled DNA ($10 \mu\text{g ml}^{-1}$) was incubated for 60 min at 37°C in the S1 assay mixture with or without S1 enzyme. The DNAs were all assayed at a concentration of $0.5 \mu\text{g ml}^{-1}$.

DNA fraction	Percentage of initial transforming activity			
	<i>purA16</i> (1.2×10^5)*	<i>leu-8</i> (6.3×10^4)*	<i>metB5</i> (6.9×10^5)*	<i>thr-5</i> (2.6×10^5)*
Native	100	100	100	100
Native + S1	93.6	82.6	88.3	84.1
HA HII	14.2	6.8	8.2	9.7
HA HII + S1	7.8	0.7	3.6	4.7

* Number of transformants ml^{-1} with native DNA assayed for the various markers.

Table 2. *Extent of S1 nuclease digestion of various fractions of DNA*

[^3H]Thymidine-labelled DNA ($2 \mu\text{g ml}^{-1}$, sp. act. $51\,200 \text{ c.p.m. } \mu\text{g}^{-1}$) and $50 \mu\text{g}$ sheared, denatured calf thymus DNA were incubated for 60 min at 37°C in the S1 assay mixture containing 0.1 M-NaCl (total vol. 2.5 ml) with 500 units of enzyme: samples (0.2 ml), in triplicate, were removed and precipitated with an equal volume of ice-cold 10% trichloroacetic acid (TCA).

[^3H]DNA fraction	Radioactivity in TCA-precipitated DNA	
	c.p.m.	% of original
Native	18 500	90
Denatured	3 100	15
MAK H	4 100	20
HA HI	3 700	18
HA HII	12 200	60

activity following thermal renaturation (68°C) has been described (Rudner & Remeza, 1973). This DNA reassociates with unimolecular kinetics, typical of intrastrand structures. Only the slowest eluting molecules of H strand DNA show an increased transforming activity following self-annealing (Fig. 1a).

Hydroxyapatite chromatography, which can separate double- and single-stranded DNA, was used to isolate the sequences which self-annealed from the rest of the DNA. Figure 1(b) shows the pattern obtained when the MAK H strand was self-annealed and eluted from a hydroxyapatite column with a linear salt gradient. The first fraction (HA HI) is entirely single-stranded while the second fraction (HA HII) contains double-stranded DNA. Only the second fraction of HA-fractionated DNA had transforming activity (Fig. 1b). Self-annealing of the L strand also produced a fraction of DNA with intrastrand structure; however, these molecules showed virtually no transforming activity. HA HII DNA showed 6 to 10% of the transforming activity of native DNA. As shown in Table 1 and previously (Rudner & Remeza, 1973), all of the markers assayed (*purA16*, *leu-8*, *metB5* and *thr-5*) were transformed by HA HII DNA, suggesting that secondary structure is located throughout the chromosome.

Molecules of DNA which are retained by hydroxyapatite (e.g. HA HII) need not be perfectly helical but can have single-stranded tails or loops (Wilson & Thomas, 1973). To determine whether the DNA sequence responsible for the transforming activity resides in the base-paired or single-stranded region of the HA HII molecules, the DNA was digested with S1 nuclease. The ionic strength is very important in controlling the specificity of this enzyme. Vogt (1973) found that S1 in 0.3 M-NaCl introduced one nick per 10 molecules of native λ DNA, whereas in 0.05 M-NaCl it introduced one nick per molecule. Similarly, the endonucleolytic activity of S1 on single-stranded DNA is greater at low NaCl concentrations. Table 2 shows the extent of degradation of various DNAs by S1 nuclease in 0.1 M-NaCl :

Table 3. Hybridization of MAK- and HA-fractionated H strands to pulse-labelled [^3H]RNA

^{32}P -Labelled DNA (10 μg ; sp. act. 16600 c.p.m. μg^{-1}) was added to a nitrocellulose filter and the amount immobilized on the filter was determined. Dried filters were incubated with 0.71 μg of pulse-labelled [^3H]RNA (sp. act. 55500 c.p.m. μg^{-1}) in $6\times\text{SSC}$ buffer at 68 °C for 16 h. DNA fractions designated D- were thermally denatured at 100 °C for 10 min prior to immobilization on filters.

DNA fraction	DNA immobilized (μg)	[^3H]RNA hybridized	
		c.p.m.	% of total
MAK H	6.7	16300	41
HA HI	9.0	9200	23
HA HII	0.8	184	0.5
D-HA HII	8.6	2900	7.3
D-HA H(I+II)	8.0	12600	32
D-HA HII+S1	6.5	2100	5.4

60% of the HA HII DNA was resistant to S1 nuclease, whereas thermal denaturation profiles (data not shown) indicated that only 25% of HA HII DNA forms helical regions, suggesting that the resistant DNA contains single-stranded, presumably unpaired, loop regions. Thus the self-complementary sequences may more closely resemble the type of structures found in plasmid DNA (Sharp *et al.*, 1973) than the hairpin structures with very small turnaround regions found in eukaryotic DNA (Wilson & Thomas, 1974). With the exception of the *leu-8* marker, all of the markers assayed were transformed at approximately 50% of their initial level by S1-treated HA HII DNA (Table 1). The *leu-8* marker is known to be highly sensitive to denaturation and to the competency of the recipients (R. Rudner, unpublished results). It is possible that the leucine gene is in a unique structural configuration that leaves it sensitive to nucleases. The retention of transforming activity by the HA HII DNA which is resistant to S1 digestion indicates that the intrastrand self-complementary sequences, or the loops between the regions, contain structural gene sequences which are directly active in biological transformation.

Hybridization to pulse-labelled [^3H]RNA

RNA extracted from bacteria which had been labelled for 3 min with [^3H]uridine while rapidly growing in the exponential phase was enriched for messenger transcripts: competition hybridization experiments showed the ribosomal RNA content to be 33% (Y. Setoguchi & R. Rudner, unpublished results). Messenger RNA is known to be transcribed asymmetrically in *B. subtilis*, with 90% hybridizing to the MAK H strand and 10% to the MAK L strand (Margulies *et al.*, 1971; Yamakawa & Doi, 1971; DiCioccio & Strauss, 1971). HA HII DNA which had been immobilized on nitrocellulose filters before or after digestion with S1 nuclease was hybridized to pulse-labelled RNA to determine the extent to which regions with secondary structure are transcribed during vegetative growth. The results of hybridization at a DNA/RNA ratio of 15:1 are given in Table 3. At this ratio, the percentage of RNA hybridized is 75% of the maximum. Thermally denatured HA HII DNA (designated D-HA HII) hybridized to pulse-labelled RNA poorly (17.7%) in comparison with the original MAK H fraction indicating that much of the mRNA that is synthesized during the exponential phase of vegetative growth is not transcribed from sequences of HA HII DNA. There was no significant loss of hybridization following hydroxyapatite chromatography since a mixture of the fractions [D-HA H(I+II)] hybridized almost (80%) as efficiently as the MAK H strand. The removal of single-strand DNA (D-HA HII+S1) did not significantly reduce the hybridization efficiency of this fraction of DNA indicating that the self-complementary sequences are transcribed at the same level as the rest of the DNA in the population.

Table 4. *Hybridization of MAK- and HA-fractionated H strands to stable [³H]RNA*

³²P-Labelled DNA (10 µg) was applied to a nitrocellulose filter and the dried filter was incubated with 0.5 µg [³H]RNA (sp. act. 20800 c.p.m. µg⁻¹) in 6×SSC buffer (total vol. 1 ml) at 68 °C for 16 h. HA HII fractions were thermally denatured at 100 °C for 10 min prior to immobilization on filters.

DNA fraction	RNA fraction	[³ H]RNA hybridized	
		c.p.m.	% of total
MAK H	23S	3200	25
HA HI	23S	1600	13
D-HA HII	23S	1100	8.7
MAK H	16S	1600	16
HA HI	16S	1600	16
D-HA HII	16S	1600	16
MAK H	5S	990	10
HA HI	5S	520	5.2
D-HA HII	5S	590	5.9
MAK H	4S	940	10
HA HI	4S	920	10
D-HA HII	4S	530	5.8

Table 5. *Sedimentation values and estimated molecular weights of native and fractionated strands of B. subtilis DNA*

Values were calculated as described in Methods.

DNA fraction	10 ¹³ × Sedimentation coefficient	10 ⁻⁶ × Molecular weight
Native	25.0	12.0
Denatured	21.9	3.5
MAK L	18.5	2.3
MAK H	21.4	3.3
HA HI	20.1	2.8
HA HII	20.2	2.9
HA HII + S1	*	1.8

* Value obtained from TCA precipitation of S1-resistant DNA.

Hybridization to isolated components of stable RNA

Margulies *et al.* (1970) have shown that transcription of stable RNA components occurs entirely off the H strand of *B. subtilis* DNA. The hybridization pattern of the isolated RNA components (23, 16, 5 and 4S) to the various H strand DNA fractions is given in Table 4. At the DNA/RNA ratio used (20:1), the percentage of RNA hybridized is maximal. Both hydroxyapatite fractions of DNA (HI and HII) showed high levels of hybridization to stable RNA indicative of the conditions of DNA excess. The 5S and 16S species hybridized equally well to both hydroxyapatite fractions while the 23S and 4S species did not. The wide range of hybridization efficiencies suggests that some portion of the ribosomal cistrons, or possibly their adjacent sequences, is composed of regions of DNA containing secondary structure.

Molecular size of fractionated DNA

The size of the DNA fractions was determined by sedimentation velocity centrifugation (Table 5). The MAK H strand fragments were larger than the MAK L fragments, consistent with the increased lability of the purine-rich L strand (Rudner & Remeza, 1973). Both hydroxyapatite fractions showed the same sedimentation coefficient for a given strand. The HA HII fraction which has reduced biological activity has a molecular weight of around 3×10^6 . Digestion of this fraction of DNA with S1 nuclease reduced its molecular weight by about 40% to greater than 1.8×10^6 .

DISCUSSION

Our studies confirm that, as in many other organisms, the DNA of *B. subtilis* contains intrastrand self-complementary sequences. The evidence that we obtained intrastrand self-complementary sequences rather than a mixture of H and L strand DNA, or cross-linked DNA, is twofold: (i) the position at which this DNA elutes from a MAK column is unusual (see Fig. 1*a*); (ii) the kinetics of reassociation are not instantaneous, as with cross-linked molecules, nor are they bimolecular, ruling out formation of L and H duplexes (Rudner & Remeza, 1973). The self-complementary sequences comprise 5 to 10% of the genome as determined by the proportion of DNA resistant to digestion with S1 nuclease.

Transformation and hybridization analyses have shown that HA HII DNA has limited biological activity. That the late-eluting H strand DNA is able to transform only after thermal renaturation reflects the well-established requirement for double-stranded DNA for penetration into a competent bacterium. The biological activity of this fraction of DNA is low; the transformation frequency is 6 to 10% of that of native DNA and the hybridization efficiency to pulse-labelled RNA is 18% of that of the MAK H strand. These data indicate that HA HII DNA contains many sequences which are not active in transformation and that much of the RNA that is made during vegetative growth does not hybridize to HA HII DNA. Of the molecules which are transcribed or have transforming activity, removal of the single-stranded portion with S1 nuclease did not substantially alter their activity indicating that regions of DNA which are self-complementary can be coding sequences for structural genes. The low transforming activity of the HA HII DNA, both before and after treatment with S1 nuclease, is not a consequence of small size. The molecular weights of the DNAs are 3×10^6 and 1.8×10^6 , respectively, well in excess of the minimum size requirement for transforming activity of 10^6 (Morrison & Guild, 1972).

Hybridization of HA HII DNA to stable RNA gave a wide range of efficiencies (between 35 and 100% of that of MAK H DNA). Doolittle & Pace (1971) have shown that rRNA molecules are derived from a transcriptional unit consisting of 16S, 23S and 5S rRNA genes in that order. Secondary structure in the spacer and promotor regions between the units may account for the greater hybridization efficiency of HA HII DNA to 16S and 5S RNA. Alternatively, differences in secondary structure of the RNAs could affect the extent of hybridization.

Whether intrastrand self-complementary DNA sequences of the type described in this study are involved in the regulation of biological processes in *B. subtilis* is in the realm of speculation. There have been many proposals (Grier, 1966; Gilbert & Müller-Hill, 1967; Sobell, 1972) that regulatory proteins, such as repressors, polymerases, recombinational enzymes etc., might recognize and bind to sequences of the type ABCC'B'A'. Self-complementary sequences with more extensive unpaired regions ABC...C'B'A' have been described in the leader sequences in both prokaryotes (Lee & Yanofsky, 1977) and animal viruses (Chow *et al.*, 1977), at the ends of insertion elements (Ohtsubo & Ohtsubo, 1978) and in several transposable DNA segments carrying antibiotic resistance genes (Kleckner *et al.*, 1975; Heffron *et al.*, 1975). It is possible that many modes of recombination occur through self-complementary sequences or that they are involved in the regulation of transcription. The studies reported here represent a first step in the isolation and characterization of intrastrand self-complementary sequences from *B. subtilis*.

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