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Identification of a suppressor sequence for DNA replication in the replication origin region of the *Bacillus subtilis* chromosome

(initiation of DNA replication/suppressor for replication/nucleotide sequence/involvement of transcription in suppression)

MOTOHARU SEIKI*, NAOTAKE OGASAWARA†, AND HIROSHI YOSHIKAWA†

*Department of Viral Oncology, Cancer Institute, 1-37-1, Kamiikebukuro, Toshimaku, Tokyo 170, Japan; and †Department of Biophysics, Cancer Research Institute, Kanazawa University, 13-1, Takaramachi, Kanazawa 920, Japan

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ABSTRACT **The first replicating fragment of the *Bacillus subtilis* chromosome, B7, inhibited the replication of the plasmid that carried this fragment. In earlier work using sequential cleavage by *Alu* I, the suppressor function was located within a 489-base-pair segment. The nucleotide sequence of the entire segment now has been determined. The sequence is characterized by two promoter-like structures and several putative recognition sequences, such as termination signals, 2-fold symmetries, inverted repeats, and repeats. By means of sequential cleavage with exonuclease BAL-31, the essential region for suppression was located in a 200-base-pair region that contains the two promoters with the same orientation. Specific transcription was produced *in vitro* by using *B. subtilis* or *Escherichia coli* RNA polymerases. The transcription was mostly from the second promoter. Elimination of the −35 region of the second promoter dramatically affected both inhibitory activity and *in vitro* transcription, suggesting that the transcriptional activity of the second promoter is involved in the *cis*-inhibition of DNA replication. The significance of the suppressor sequence in the region of the replication origin of the *B. subtilis* chromosome is discussed.**

Initiation of chromosomal replication in bacteria is strictly regulated in order to synchronize the rate of synthesis of genetic material with the growth rate. Recent studies with *Escherichia coli* have revealed that functions required for autonomous replication and regulation are clustered in the replication origin region of the chromosome (1–3). In *Bacillus subtilis*, no DNA segment responsible for the initiation of chromosomal replication has been identified because of the failure to isolate an autonomous replicative sequence from the chromosome. Recently we reported the identification, in the region of the chromosomal replication origin, of a segment that inhibits replication of plasmids in *B. subtilis* when the segment is inserted in plasmid DNA (4). The inhibition acted in *cis*-configuration; thus, the segment inhibited the replication of the plasmid that carried it but did not inhibit that of the parental plasmid coexisting in the same cell (4). By using a sequential deletion from either end of the segment, we were able to limit the essential region within 500 base pairs (bp) (5).

In this report we present the nucleotide sequence of the minimal region essential for the inhibitory function. *In vitro* transcription from this region suggests a direct involvement of transcription in the inhibition. The role of the suppressor sequence in the regulation of initiation of the chromosomal replication is discussed.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. *B. subtilis* RM141 (*arg*15, *leu*18, *his*A1, *hsr*M, *hsm*M, *rec*E4, a gift from T. Beppu, University of Tokyo) was used as a recipient for plasmid transformation. *E. coli* C600 (*thr*, *leu*, *thi*, *ton*A, *lac*, *sup*F) was used for cloning. Plasmids pMSd208 and pMSd117, containing portions of the first replicating fragment of the *B. subtilis* chromosome, were described in the previous paper (5). The vector plasmid pMS102′, already described (4), is composed of pBR322 [ampicillin-resistant (Amp^r)] and pUB110 [kanamycin-resistant (Km^r)] and confers both Amp^r and Km^r to *E. coli* and Km^r to *B. subtilis* by transformation. Partial deletions from pMSd208 by exonuclease BAL-31 is described below.

Chemicals and Enzymes. [α-^{32}P]dCTP (PB10205) and [α-^{32}P]ATP (PB10160) were from Radiochemical Centre (Amersham, England). The *Bam*HI and *Hin*dIII linkers, T4 DNA ligase, and all restriction endonucleases were from Takara Shuzo (Kyoto, Japan). DNA polymerase I (*E. coli*) and BAL-31, and T4 polynucleotide kinase were from New England BioLabs.

Nucleotide Sequence Determination. The nucleotide sequence of the fragment was determined by the method of Maxam and Gilbert (6). The 3′ ends were labeled by incorporating [α-^{32}P]dCTP by DNA polymerase I into cohesive ends produced by *Bam*HI or *Hin*dIII. The reaction mixture contained 50 mM Tris·HCl (pH 7.8), 100 mM KCl, 7 mM $MgCl_2$, 7 mM dithiothreitol, 0.4 mM dATP, dGTP, and dTTP, 50 μCi (1 Ci = 3.7×10^{10} becquerels) of [α-^{32}P]dCTP (2,000–3,000 Ci/mmol), 1.2 pmol of DNA fragment, and 1 unit of DNA polymerase I in 50 μl. The incubation was at 12°C for 30 min.

Preparation of Deletions by BAL-31. The procedure is shown schematically in Fig. 3. Plasmid pMSd208 contained a *B. subtilis* origin fragment of ≈2,840 bp with a *Bam*HI site at the left end and a *Kpn* I site at about 800 bp from the *Bam*HI site (see Fig. 3). The plasmid DNA was linearized with *Bam*HI or the combination of *Kpn* I and *Hin*dIII. The linear DNA (4–5 μg) was then digested with BAL-31 in a buffer specified by the producer's manual at 37°C. In this condition, 0.06 unit of enzyme cleaved 10 bp in 1 min, and 1.5 units cleaved 500 bp in 2 min. The resultant "nibbled ends" were joined to molecular linkers. An ≈440-fold excess of 5′-P linker (phosphorylated by T4 polynucleotide kinase) was used to ligate (with T4 DNA ligase) to blunt ends of the nibbled DNA. To produce cohesive ends of linker molecules, the linked DNA was cleaved with either *Bam*HI or *Hin*dIII before circularization with T4 DNA ligase. The mixture of the circular DNA was used to transform *E. coli* for Amp^r and each transformant was used to clone each deleted plasmid in the presence of chloramphenicol (Cm) as described (4).

***In Vitro* Transcription.** The reaction mixture for *B. subtilis* RNA polymerase contained 100 mM Tris·HCl (pH 7.6), 2 mM $MnSO_4$, 4 mM $MgCl_2$, 0.4 mM dithiothreitol, 120 mM KCl, 5% (vol/vol) glycerol, 100 μM CTP, UTP, and GTP, 1 μM

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Abbreviations: Km, kanamycin; Amp, ampicillin; Cm, chloramphenicol; bp, base pair(s).

[α-^{32}P]ATP (400 Ci/mmol), 1 pmol of DNA fragment, and RNA holoenzymes (0.25–0.3 μg) in 50 μl. *B. subtilis* enzymes containing σ_{55}, σ_{37}, or σ_{29} subunit (7) were donated by K. Nakayama (Miyazaki Medical School). For the *E. coli* enzyme the reaction mixture was 40 mM Tris·HCl (pH 8.0), 150 mM KCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, 10% glycerol, 200 μM CTP, UTP, and GTP, 1 μM [α-^{32}P]ATP (400 Ci/mmol), 1 pmol of DNA, and 0.5 μg of RNA polymerase in 50 μl. The *E. coli* holoenzyme was a gift from A. Ishihama (Kyoto University). Transcripts were extracted by phenol treatment and precipitated with tRNA as a carrier. Electrophoresis was in 6% polyacrylamide gel buffered with 7 M urea/90 mM Tris borate/2.5 mM EDTA at 1,000 V for 8 hr.

Plasmid Transformation. Transformation and amplification of plasmid in *E. coli* were as described (4). Transformation of *B. subtilis* cells by plasmid was carried out according to a protoplast fusion-regeneration method described by Chang and Cohen (8).

RESULTS

Nucleotide Sequence of the 500-bp Region. The suppressor function for plasmid replication was first localized in the first replicating fragment, *Bam*HI-7 (B7), of the chromosome (4). The 5.4-kbp B7 fragment was then cleaved sequentially by *Alu* I to determine the location of the suppressor function within the two neighboring *Alu* I sites (5). This region was estimated to contain ≈500 bp.

Fig. 1 shows the scheme of the sequence determination. To determine terminal portions of the segment, two *Bam*HI sites—produced by rejoining *Bam*HI sites of the vector plasmid and *Alu* I site of the segment after repairing cohesive ends by DNA polymerase I (5)—were used to label the terminal nucleotides, and both strands were used for sequence determination. In order to determine the sequence of the center portion of the segment, a series of plasmids was constructed with deletions in either terminal portion of the segment. The isolation of the deletion is described in the next section. In addition to the sites shown in Fig. 1, 26 additional sites shown in Fig. 2 were used to establish the sequence in the center portion of the segment.

The segment was found to consist of 489 bp and shows the following characteristic features. The first 313 bp are A+T-rich (69%) and contain many repeats of 6–12 A·T pairs; the rest of the segment is rich in G+C (57%). There are two promoter-like structures of the same orientation located 98 bp apart. Both promoter-like structures contain a typical Pribnow's box and −35 region specificities and act as promoters for *B. subtilis* and *E. coli* RNA polymerases as shown below.

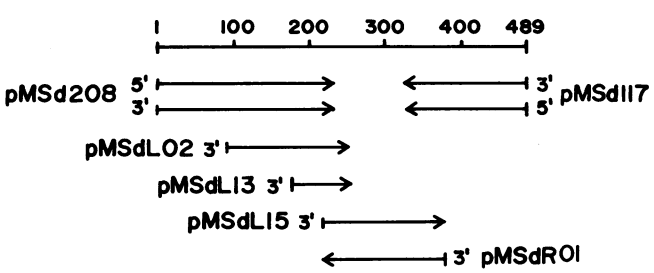

FIG. 1. Strategy for determination of nucleotide sequence of the 489-bp inhibitory segment. Arrows represent orientation and size of the sequence determined by using the individual plasmid indicated. Plasmids pMSd208 and pMSd117 contained one *Bam*HI site at the junction of the segment to the vector plasmid (positions 1 and 489, respectively) as described (5). The pMSdL series are derivatives from pMSd208 and contain deletions from the *Bam*HI site. Each plasmid has one newly constructed *Bam*HI site at the junction to the vector. pMSdR01 is a derivative of pMSd208 and contains one *Hin*dIII site at the rightmost junction to the vector. Construction of the deletion plasmids is shown in Fig. 3 and Table 1. Additional sites indicated in Fig. 2 were used to determine the sequence of the center portion of the segment.

Table 1. Transforming activity of plasmids containing various portions of the origin fragment

Plasmid	Remaining fragment of B7*	Relative no. of transformants†	Activity class‡
pMSd100	0	200–1,000	++
pMSd208	1–2,840	0	−
pMSdL02	85–2,840	0	−
pMSdL03	86–2,840	0	−
pMSdL04	92–2,840	0	−
pMSdL05	100–2,840	0–3	±
pMSdL06	101–2,840	0–3	±
pMSdL07	106–2,840	1–4	±
pMSdL08	108–2,840	**2**	±
pMSdL09	109–2,840	1–15	±
pMSdL10	115–2,840	0–1	±
pMSdL11	142–2,840	0–41	+
pMSdL12	154–2,840	**13**	+
pMSdL13	174–2,840	0–12	+
pMSdL14	212–2,840	400–1,000	++
pMSdL15	220–2,840	250–1,000	++
pMSdL16	(245–255)–2,840	**200**	++
pMSdL17	(245–255)–2,840	**232**	++
pMSdR01	1–380	0	−
pMSdR02	1–378	0	−
pMSdR03	1–350	0	−
pMSdR04	1–326	0	−
pMSdR05	1–317	**25**	+
pMSdR06	1–316	**1**	±
pMSdR09	1–283	3–53	+
pMSdR10	1–274	**1**	±
pMSdR11	1–206	**664**	++
pMSdR13	1–(194–204)	49–400	++
pMSdR17	1–179	200–500	++
pMSdR18	1–168	200–500	++
pMSdR20	1–130	**500**	++

* Numbers represent positions of nucleotide shown in Fig. 2. The number 2,840 is an approximate position of the right-hand end of the B7 fragment. In the case of the pMSdR series, remaining rightmost portions of B7 segment are neglected (see Fig. 3). Numbers in parentheses indicate that the site is ambiguous within the range indicated.

† Values show the range of variation in more than two independent experiments. For each experiment, an average of at least three plates was determined. Values within **boldface** are from one experiment and are the average of at least three plates.

‡ −, Less than 1/1,000 of the vector plasmid pMSd100 and similar to the parental plasmid pMSd208; ±, about 1/100 of the vector plasmid; +, about 1/10 of the vector plasmid; ++, about the same as the vector plasmid.

Two stable palindromic structures can be formed, one 12 bp upstream and the other immediately after the first promoter sequence. The former resembles a termination signal for the transcription. Two 10-bp stretches (identical except for one extra G·C pair), A-A-G-A-C-A-C-A-A-G, are found exactly 20 bp downstream from each Pribnow's box. The first stretch constitutes the major part of the stem of the palindrome located immediately after the first promoter. In addition, many putative signal sequences such as repeats and inverted repeats are clustered in this region.

Determination of the Essential Sequence for the Suppressor Function. The minimal sequence essential for the inhibitory activity was determined by constructing a series of sequential deletions from either end of the segment (Fig. 3). Because there

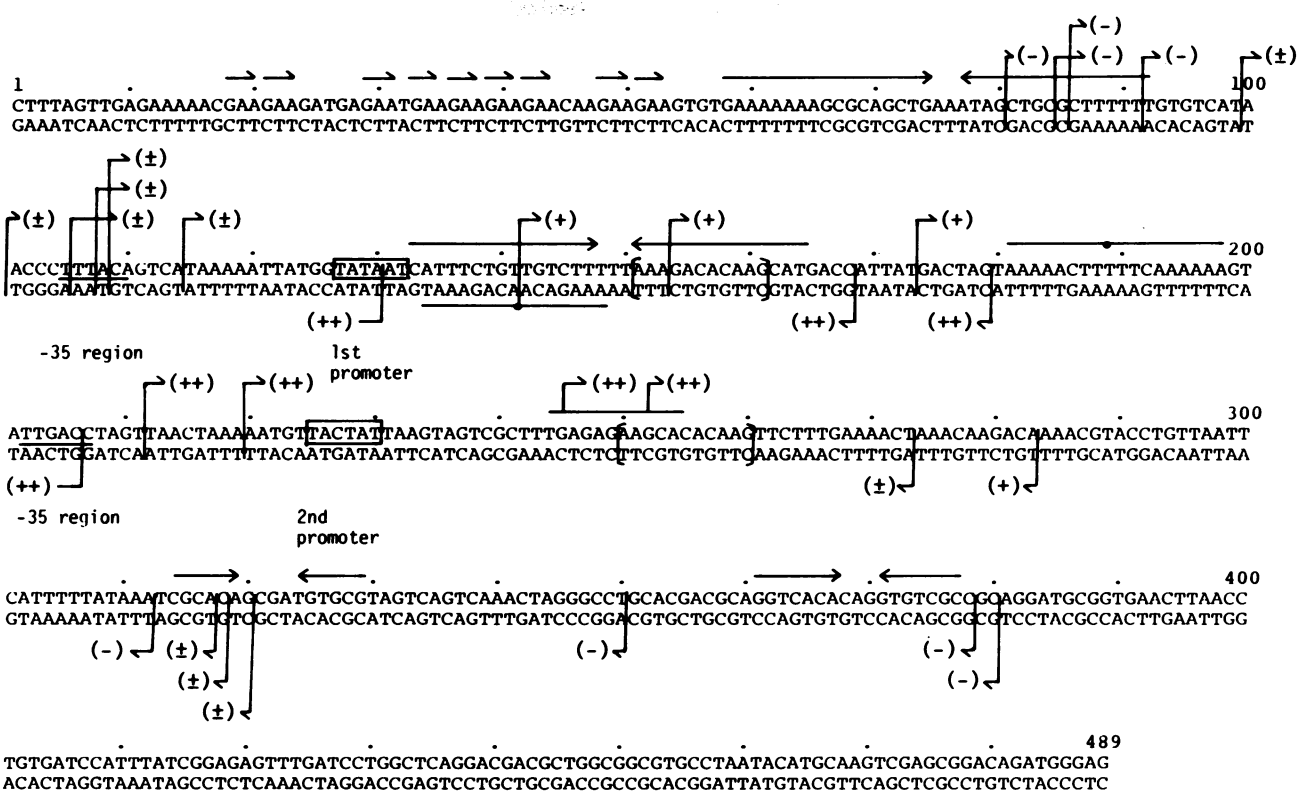

FIG. 2. Nucleotide sequence of the 489-bp segment and the essential region for the inhibitory function (*sar* sequence). Nucleotide sequence of the entire 489-bp segment is shown. →, Repeated sequence; → ←, 2-fold symmetry; —•—, inverted repeat; □, Pribnow's box; [ ], unique 10-bp repeat. In order to show the essential region for the inhibitory function, the terminal nucleotide of the remaining fragment in each deleted plasmid and transforming activity of deleted plasmid are indicated along the sequence as follows: ↱, sequence is deleted from the left up to this site; ↲, sequence is deleted from the right up to this site. In parentheses, transforming activity classes are shown as indicated in Table 1 and in the text.

were no appropriate cleavage sites for restriction enzymes, the double-stranded DNA was digested sequentially with an exonuclease, BAL-31, to various extents. The parental plasmid, pMSd208, contained a B7 fragment whose left end corresponds to nucleotide 1 of the 489-bp segment (see Fig. 2). The vector plasmid is a composite plasmid of pBR322 and pUB110 and can replicate in both *E. coli* and *B. subtilis*. It confers resistance to Amp and Km to *E. coli* and to Km to *B. subtilis* cells, respectively. Insertion of the fragment containing the inhibitory region decreased the transformation of *B. subtilis* Km^r to less than 1/1,000. It also induced the loss of the plasmid from *E. coli* when selective pressure for the plasmid was not present.

When the segment was deleted from left to right, the *Bam*HI site at the nucleotide 1 was used to make a linear molecule. After digestion with BAL-31 to various extents from the *Bam*HI site, circular molecules were restored by using *Bam*HI linkers. When the segment was deleted from right to left, a neighboring *Kpn* I site (about 800 bp to the right of the *Bam*HI site) was used. In this case the parental plasmid was cleaved by *Kpn* I and *Hin*dIII simultaneously to remove most of the B7 fragment unnecessary for the inhibitory function. *Hin*dIII linker was used to restore a circular plasmid molecule. Each molecule was cloned in *E. coli* and its nucleotide sequence was determined from the newly inserted linker sites to determine the location of the deletion within the 489-bp segment. By this method, 17 deletions from the *Bam*HI site and 13 deletions from the *Kpn* I site were isolated (Table 1; Fig. 2).

The efficiency of transformation of *B. subtilis* to Km^r by these deletion plasmids was measured by comparison to the parental and vector plasmids. The results are summarized in Table 1 and Fig. 2. Because efficiency of transformation by the protoplast method varies even in different plates of the same experiment, the activities by the deletion plasmids were classified into four groups. Under the present assay conditions, no transformants were detected by the parental plasmid containing the intact 489-bp segment. When the deletion proceeded from the *Bam*HI site (left to right) up to the −35 region of the first promoter-like structure, a small number of transformants, corresponding to 1/1,000 of the vector plasmid, started to appear. Transforming activity increased gradually as the deletion proceeded, to reach the level of the vector plasmid when the −35 region of the second promoter-like structure was deleted. When the deletion occurred from the *Kpn* I site (right to left), elimination of the second promoter was sufficient to remove the inhibitory function completely.

We conclude that the essential region for the suppressor function is a 220-bp sequence from nucleotides 100 to 320. The minimal essential region can be more strictly limited to a region of 120 bp around the second promoter. The only signal sequences in the essential region are two promoter-like structures. In both cases, deletion of the −35 region of the promoters affected the inhibitory function of the segment. The second of the two was the most effective because deletion of its −35 region completely eliminated the inhibitory effect. These results strongly suggest that the promoter activity, mostly of the second promoter and to some extent of the first promoter, is involved in the *cis*-inhibition of DNA replication by the segment.

Promoter Activities of the Essential Region *in Vitro*. Transcriptional activity by the two putative promoters in the essential region was determined *in vitro* by using RNA polymerases from *B. subtilis* and *E. coli*. An 800-bp DNA segment containing the essential region within the leftmost 300 bp of the segment

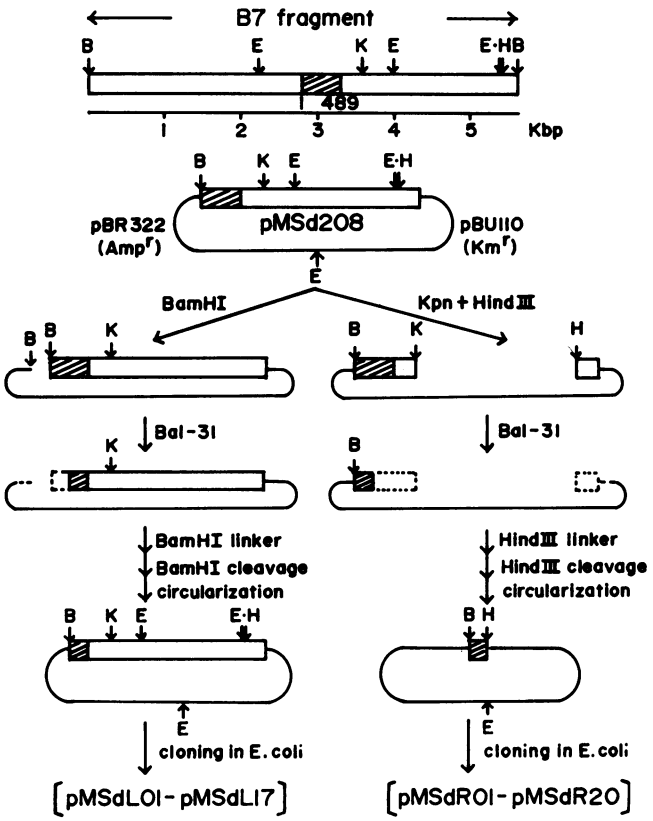

FIG. 3. Construction of the deletions from the 489-bp segment. At the top, the first replicating segment B7 containing the 489-bp suppressor segment (shaded area) is shown. Restriction enzyme cleavage sites are shown by arrows: B, *Bam*HI; E, *Eco*RI; K, *Kpn* I; H, *Hind*III. Plasmid pMSd208 contains about half of the B7 fragment. *Bam*HI site of the rightmost end of the B7 was eliminated and the new *Bam*HI site was constructed at nucleotide 1 of the 489-bp segment as described (5). Broken lines indicate the segment cleaved by BAL-31. During the digestion with BAL-31, equivalent amounts of nucleotides were also cleaved from the vector or remaining part of the B7 fragment as shown. The loss of these segments alone has no effect on the replication of the plasmid (5). In the figure, the size of the vector portion is arbitrary.

was prepared and used as a template. Transcripts from the first and second promoters should be 700 and 600 nucleotides long, respectively. In addition, deletion plasmids described above were used to isolate the same 800-bp segment containing various deletions at the two promoter regions (Fig. 4).

Major transcripts by *B. subtilis* RNA polymerase holoenzyme (vegetative form σ_{55}) were about 600 bp long (Fig. 4 *Left*). Deletion of the −35 region of the second promoter completely eliminated the synthesis of such transcripts; deletions of the first promoter had no effect. Therefore, a specific transcription occurred from the second promoter. The first promoter is scarcely used by the enzyme. Furthermore, no other promoter sites were detected within the 800-bp segment. RNA polymerase holoenzymes containing σ_{29} or σ_{37} showed essentially the same results except that the level of transcription was less than 1/10 of that with the σ_{55} enzyme.

Slightly different results were obtained with *E. coli* RNA polymerase holoenzyme (Fig. 4 *Left*). In addition to the transcription from the second promoter, a weak transcription occurred from the first. A deletion in nucleotide sequence near the −35 region enhanced the transcription of the first promoter (lane 2). Both promoters were shown to be specifically bound by the *E. coli* RNA polymerase by using a DNase detection method (data not shown).

DISCUSSION

We have identified, within the region of the replication origin of the *B. subtilis* chromosome, a DNA sequence that suppresses the autonomous replication of plasmids in the cell. We term this sequence "*sar*" for *s*uppressor for *a*utonomous *r*eplication. The sequence was first found in the first replicating 5.4-kbp fragment of the chromosome (4). By stepwise deletions from either end of the fragment by *Alu* I, the functional region was located to within the two neighboring *Alu* I sites containing ≈500 bp (5). Finally, by the sequential cleavage with exonuclease BAL-31 from either end of the segment, the essential region was limited to a sequence of 200 bp. During the course of the last deletion experiment, we cloned a 300-bp fragment which contained the essential region and we demonstrated that the suppressor function indeed resides solely in this fragment.

Because no peptide can be synthesized from the essential region, it is unlikely that any product of the region affects the replication of the plasmid. This is consistent with the fact that the suppression does not take place against other molecules but, in *cis*, occurs against the same molecule that carries the sequence (4). The sequence is characterized by two promoter-like structures and a symmetrical sequence rich in A+T (74%) between the two promoters. Elimination of the −35 region of the second promoter had dramatic effects on both the suppressor activity and *in vitro* transcription. We therefore conclude that the transcription from, or binding of RNA polymerase to, the second promoter is involved in the *cis*-inhibition. So far, no direct evidence is known for the involvement of protein factors in the suppression. However, the fact that the sequence inhibited plasmid replication strongly in *B. subtilis* but only weakly in *E. coli* (4) suggests the involvement of proteins synthesized in *B. subtilis* in the inhibition.

The precise location of the initiation site of the *B. subtilis* chromosomal replication has not been identified. We have reported that initiation started bidirectionally from within a 5.4-kbp fragment of B7 (9, 10). Although the *sar* sequence is also found within the same fragment, the relative locations of these two sites are unknown. However, isolation of autonomously replicating fragments from the *B. subtilis* origin by random-type cloning experiments has been unsuccessful (4). In addition, none of the cloned fragments located near the first replicating fragment showed autonomous replication (4). Furthermore, partial deletion of the fragment from either end did not produce replicating fragments (5). These results strongly suggest that the sequence required for the autonomous replication is located close to or overlaps the *sar* sequence.

Recently, in collaboration with S. Seror-Laurent (Universite de Paris-Sud), the B7 fragment was found to contain a sequence homologous to ribosomal RNA genes (unpublished data). Comparison of restriction enzyme cleavage sites of the B7 fragment with those of ribosomal DNA (11) suggests strongly that the promoters in the *sar* sequence are the promoters for the ribosomal RNA operon itself. Because the B7 is the first replicating fragment of the *B. subtilis* chromosome, the ribosomal RNA operon in the B7 fragment may correspond to a ribosomal RNA operon, *rrn*A, recently discovered near the replication origin (12).

Regulation of initiation by *sar* sequence is a negative control device. In most of the cell cycle, initiation of the chromosomal replication is in a state suppressed by the sequence. The suppression may be removed by a yet unknown mechanism to initiate replication in harmony with cell growth. This activation mechanism seems also to act in *cis* because the replication of the plasmid with the *sar* sequence is inhibited throughout the cell cycle. Structure and function of some neighboring region

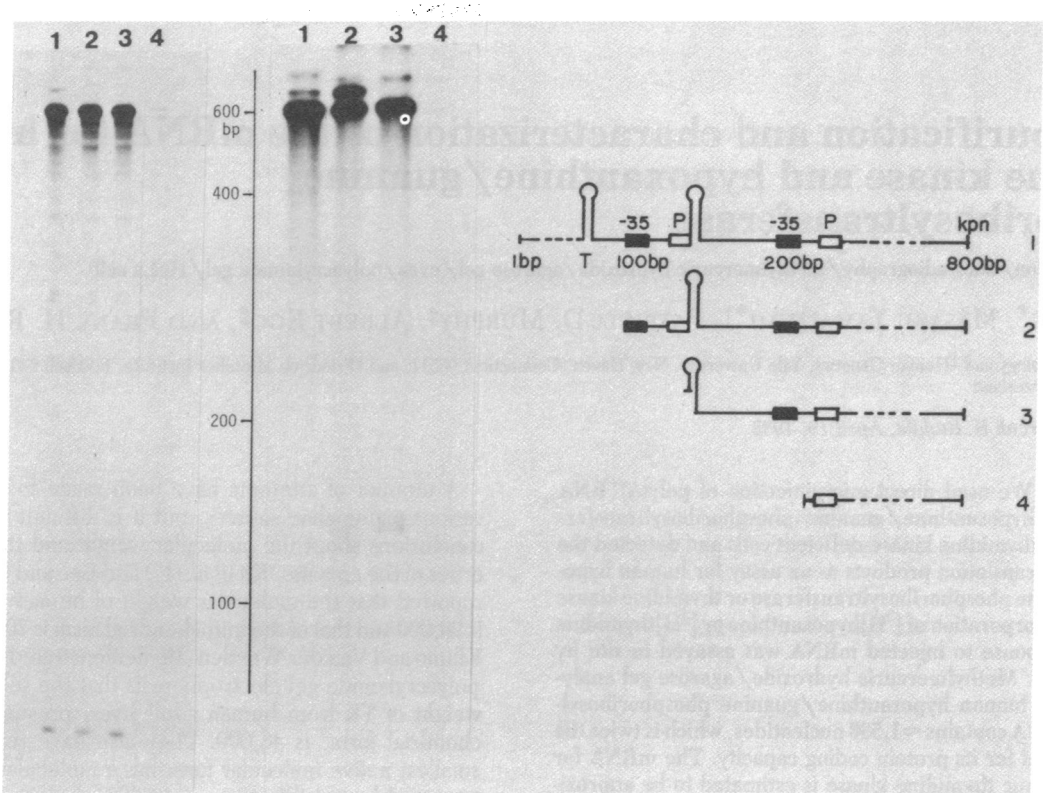

FIG. 4. *In vitro* transcription of the essential region. (*Right*) Templates used for the *in vitro* reaction, shown schematically. Two promoter-like structures, Pribnow's box (P), −35 region, and palindromes are indicated. Template 1: fragment from pMSd208, from nucleotide 1 of the 489-bp segment (Fig. 2) to *Kpn* I site of the B7 fragment (Fig. 3). Template 2: fragment from pMSdL05, from nucleotide 100 to *Kpn* I site. Template 3: fragment from pMSdL11, from nucleotide 142 to *Kpn* I site. Template 4: fragment from pMSdL15, from nucleotide 220 to *Kpn* I site. (*Left*) Autoradiogram of the transcripts separated by electrophoresis in acrylamide gel. The lane number corresponds to the number of the template used. First set: *B. subtilis* RNA polymerase σ_{55} enzyme. Second set: *E. coli* RNA polymerase holoenzyme.

of the chromosome may be important in this regard. One candidate for such a region is the site that forms a specific DNA–protein complex located next to the replication origin (13). The formation of the complex is known to be closely related to the cell's ability to initiate replication of the chromosome (14). During cloning we removed the segments adjacent to the suppressor sequence. This procedure would consequently result in loss of the mechanism of activation.

We express our appreciation to Dr. K. Nakayama for the gift of *B. subtilis* RNA polymerase and generous offer to use his facilities for the *in vitro* transcription experiments. We also thank Dr. A. Ishihama for the generous supply of *E. coli* RNA polymerase. We appreciate the generous advice of Dr. H. Ohmori on DNA sequence determination. We thank Dr. T. H. Jukes for critical reading of the manuscript. We thank Dr. K. Yamaguchi and other colleagues in our laboratory for discussion and Mrs. K. Terada and Miss K. Ando for their help. This work was supported by a Grant-in-Aid for Special Project Research, for Cancer Research, for Cooperative Research, and for Scientific Research from the Ministry of Education, Science, and Culture, Japan.

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