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Sequence limits of DNA strands in the arrested replication fork at the *Bacillus subtilis* chromosome terminus

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ABSTRACT

The DNA sequence limits of the leading and lagging strands in the arrested clockwise replication fork at the terminus of the *Bacillus subtilis* chromosome have been investigated. On the basis of hybridization to synthetic oligonucleotides corresponding to known positions in the terminus region sequence it has been shown that neither the leading nor lagging strands, as they approach *terC*, traverse the distal inverted repeat, IRI. But a small fraction of the leading strands pass through the proximal inverted repeat, IRII. This is consistent with IRI being the functional inverted repeat in arresting the clockwise fork. But most of the forks appear to stop at least 100 nucleotides short of IRI, and at various positions extending over a distance of at least 100 nucleotides.

INTRODUCTION

The first stage in termination of chromosome replication in *Bacillus subtilis* involves arrest of the clockwise fork at a sequence specific site, *terC* (1,2,3). The anticlockwise fork does not arrive at *terC* to complete replication until a few minutes later and therefore it has been possible to identify the region of DNA within which termination occurs. The region spanning *terC* is characterized by the presence of two imperfect inverted repeats, IRI and IRII (47 and 48 nucleotides respectively) separated by 59 nucleotides (3), which has been called the inverted repeat region, or IRR. On the basis of the effect of DNA deletions and modification in the vicinity of the IRR on fork arrest it was suggested that a protein (called RTP, for replication terminator protein) encoded by the *rtp* gene adjacent to the IRR binds to the IRR (or *terC*) to cause arrest of the clockwise fork (4). The relevant features of the terminus region of the *B. subtilis* chromosome are shown in Fig. 1. The clockwise replication fork enters this region from the right, proceeding through *rtp* towards the IRR.

The arrested fork is shown at the bottom of Fig. 1. The RTP protein has been isolated; it binds to the IRR as proposed (5). More recently DNase

footprinting experiments have established that it recognises specifically segments of sequence within the IRR corresponding approximately to IRI and IRII (P.J. Lewis and R.G. Wake, in preparation).

At the time of establishing the presence of the inverted repeats at *terC* in *B.subtilis* and raising the possibility that they were involved in fork arrest it was noted that the *Escherichia coli* plasmid R6K terminus region (6) also contained a pair of inverted repeats (3). It is now known that each of the R6K inverted repeats binds a protein to block replication fork movement in only one or the other direction (7,8,9). It has been suggested that the R6K repeats are orientated such that a single fork (clockwise or anticlockwise) can pass through the first repeat encountered, but it stops at the second. This would explain why a replication fork approaching the R6K terminus from either direction is arrested (10).

It is not yet known if each of the inverted repeats at the *B. subtilis* chromosome terminus functions in a manner analagous to those in R6K. But if this is the case it would be reflected in the sequence limits of the newly synthesized strands within the arrested clockwise fork. Thus, newly synthesized strands might pass through the first inverted repeat encountered (IRII) but not the second (IRI). Previously it was found that the ends of the new strands at the fork junction appeared to be distributed over a relatively broad distance (up to approximately 100 nucleotides) and closer to IRII than IRI. In this paper we examine more directly the sequence limits of the new strands (leading and lagging) as they occur in the clockwise fork arrested within the *B. subtilis* chromosome.

MATERIALS AND METHODS

Bacterial strain and plasmid

B. subtilis strain GSY1127, a class II stable merodiploid (*hisH2 ilvC/ ilvC*$^{+}$) was obtained from C. Anagnostopoulos. Plasmid pWC1 contained the 1.1 kb *Eco*RI-*Hind*III portion of the *terC* region (3) cloned in the Bluescript vector SKM13+.

DNA preparations

For the RNA probing of leading and lagging strands the DNA was the same as that used previously (3). For oligonucleotide probings the DNA from exponentially growing GSY1127 was extracted by the osmolysate procedure (3) using either *Bam*HI or a mixture of *Bam*HI + *Eco*RI. These extracts were

then deproteinized with phenol, the DNA precipitated with ethanol and dissolved in 0.01M Tris-HCl (pH 8.0) - 0.001M EDTA.

RNA and oligonucleotide hybridization probes

Radioactive RNA transcripts were synthesized *in vitro* according to the high specific activity protocol of Stratagene Cloning Systems. *Eco*RI- and *Hin*dIII-cleaved pWC1 preparations were used as the DNA template for T7 and T3 RNA polymerase, respectively. The specificity of each of the ^{32}P-RNA probes for just one strand of DNA was checked by hybridization to complementary strands from the terminus region resolved from one another in a neutral gel after strand separation in alkali (11). Oligonucleotides were made using an Applied Biosystems Model 381A DNA Synthesizer. They were labeled at the 5'- end with ^{32}P using T4 polynucleotide kinase and separated from unincorporated nucleotides by standard procedures (11).

Agarose gel electrophoresis, transfer of DNA to membranes and hybridization

One dimensional electrophoresis in alkaline agarose gels and transfer to nylon membranes was as described previously (3). For probing with ^{32}P-labeled oligonucleotides ~10 μg DNA were loaded into each lane in order to give sufficient signal after fluorography. When the *Bam*HI + *Eco*RI-cleaved DNA was to be probed by oligonuclotides, electrophoresis was allowed to proceed over only 60-70% of the distance of that achieved previously (3) in order to keep the ~1.2 kb band more compact. The conditions for neutral and subequent alkaline electrophoresis of the isolated forked DNA from a *Bam*HI digest were the same as described previously (2) except for the high DNA loading. The conditions for hybridization with RNA probes were as recommended by Stratagene Cloning Systems. Prehybridization with ^{32}P-labeled oligonucleotides was in 10 x Denhardt's solution (see ref.11) 6 x SSC, 50mM sodium phosphate (pH 6.8), 1% SDS, 1mM EDTA, 100μg/ml sonicated herring sperm DNA (freshly denatured) at 42°C. Hybridization was in the same solution, less Denhardt's solution and heterologous DNA. The temperature of hybridization was 18-22°C below the estimated Tm (see ref.12). Membranes were washed according to the protocol of Wood *et al.* (13) using tetramethylammonium chloride to give sequence independent hybridization. In 3.0M tetramethylammonium chloride at the washing temperature of 65°C, only those oligonucleotides bound by 23 or more base pairs should remain hybridized to the membrane. Exposure of the membranes to X-ray film utilized fluorography (2 Cronex Hl-PLUS intensifying screens) at -80°C for 1-14 days.

RESULTS

Low resolution analysis of leading and lagging strand termination in the arrested clockwise fork

The arrested clockwise fork is readily detected in appropriate digests of DNA isolated from exponentially growing cells of the merodiploid strain of *B. subtilis*, GSY1127 (2). (The merodiploid strain has the advantage of yielding larger amounts of the arrested fork.) It migrates more slowly than a linear fragment from the same segment of DNA. When analysed by gel electrophoresis in alkali the terminated leading and lagging strands of the forked molecule migrate faster than the larger full-length strands (14). Digestion with *Bam*HI + *Eco*RI gives a forked molecule which yields full-length strands of 10.9 kb and shorter leading and lagging strands of ~1.2 kb (Fig. 1). It was observed previously, by alkaline electrophoresis in agarose gels, that the strands of ~1.2 kb tended to resolve into two sub-species covering a range of ~100 nucleotides (3). The possibility that the two sub-species represented the leading and lagging strands had not been explored. In the present work, the same DNA samples, after electrophoresis in alkaline gels and transfer to membranes, were hybridized with ^{32}P-RNA probes for the leading and lagging strands respectively. No significant difference was found in the two cases with respect to the strands of ~1.2 kb (data not shown). Thus, the broad distribution of single strands of ~1.2kb observed previously and the tendency to resolve into two sub-species was not due to partial resolution of leading and lagging strands.

Oligonucleotide probing to establish the limits of the terminated leading and lagging strands

Fig. 2 defines the sequence locations of 10 oligonucleotides (25mers) used as probes for the leading and lagging strands of the arrested fork. The bracketed section in Fig. 2 indicates the segment within which termination of most leading plus lagging strands was found to occur in previous low resolution studies (3). The sequence limits of the leading and lagging strands were investigated here by hybridizing the ^{32}P- labeled oligonucleotide probes to *Bam*HI + *Eco*RI-digested DNA from exponentially growing GSY1127 which had been electrophoresed in an alkaline gel and transferred to a nylon membrane. Probing of an arrested fork contained within a *Bam*Hl + *Eco*Rl fragment should show the two single strand species, of 10.9 and ~1.2 kb. The former will be much larger in amount because in most cases the 10.9 kb segment of the chromosome does not contain a fork.

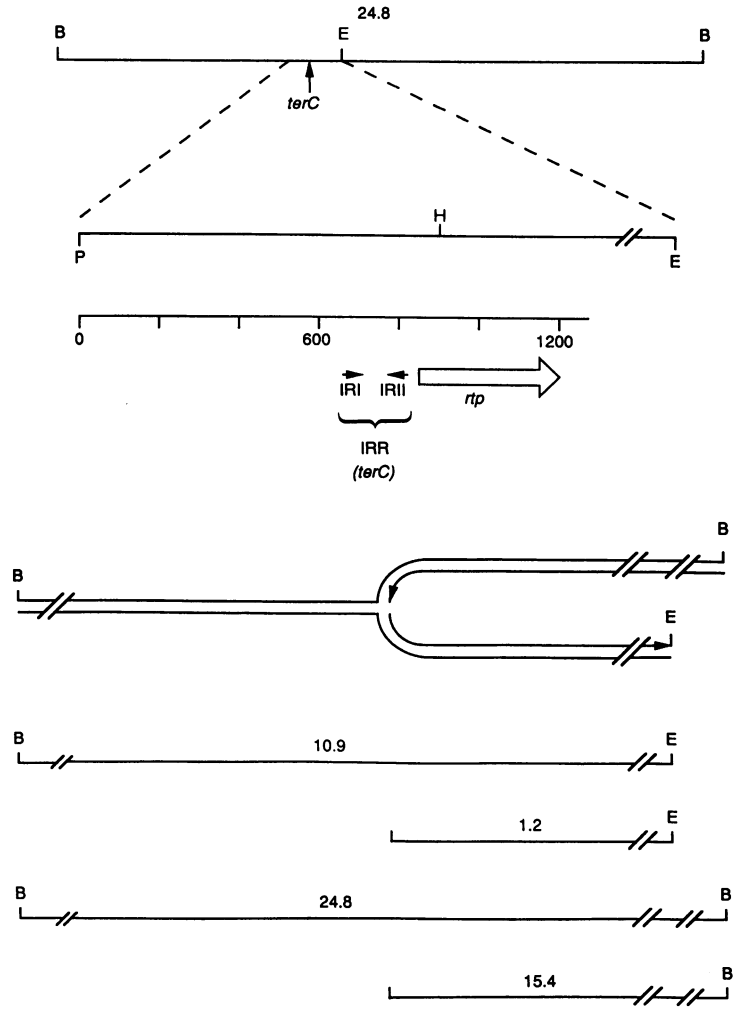

Fig.1. Features of the replication terminus region of the *B.subtilis* chromosome and the clockwise fork arrested at *terC* . *terC* is located within a *Bam*HI segment of 24.8 kb and approximately 1.2kb to the left of an internal *Eco*RI site (uppermost map). The sequence of 1267 nucleotides spanning *terC* was determined previously (3). Within this sequence the two inverted repeats (IRI and IRII), which together make up the IRR, lie just upstream of the *rtp* gene (open arrow). The clockwise fork enters this region of the chromosome from the right and stops in the vicinity of the IRR (which would correspond to *terC*). The lower poriton of the Fig. shows the dimensions of the arrested fork derived from the *Bam*HI + *Eco*RI and *Bam*HI segments of the chromosome. The arrowheads in the fork indicate the 5'⟶ 3' direction of the leading (upper) and lagging (lower) strands. B, *Bam*HI; E,*Eco*RI; H,*Hin*dIII; P,*Pst*I. Sizes are in kb.

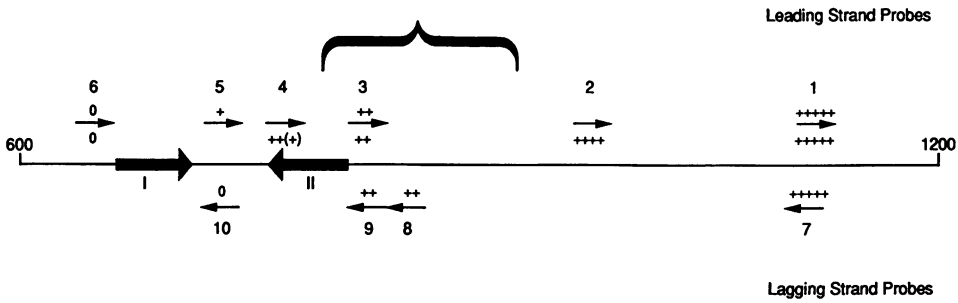

Fig. 2. Location of oligonucleotide probes within the terminus region sequence and a summary of the hybridization data from all experiments. The segment of DNA shown covers the 600-1200 nucleotide portion of the 1267 nucleotide region previously sequenced (see Fig.1). IRI and IRII are defined by heavy, filled arrows. The smaller arrows define the locations and 5′⟶ 3′ directions of the oligonucleotides used as probes for the leading strand (1-6, above sequence) and lagging strand (7-10, below sequence) of the arrested fork. The sequence positions (inclusive) of the oligonucleotides are as follows : 1,1105-1129; 2,959-983; 3,814-838; 4,761- 785 ; 5,721-745; 6,635-659; 7, 1124-1100; 8,863-839; 9,838-814; 10,745-721. The plusses (+) associated with each oligonucleotide indicate the relative semiquantitative level of hybridization achieved with each probe in various experiments. Those above the oligonucleotide positions (small arrows) were obtained from the examination of *Bam*HI + *Eco*RI digests of DNA (Figs. 3 and 4) and those below (leading strand probes only) from examination of forked DNA from a *Bam*HI digest (Fig.5).

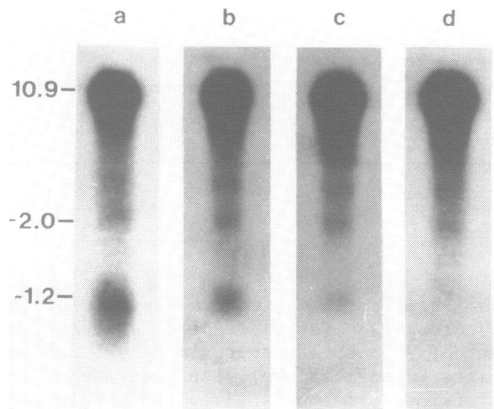

Fig.3 Autoradiographs of *Bam*HI + *Eco*RI digests of DNA obtained from exponentially growing GSY1127 and fractionated by gel electrophoresis in alkali. Each panel was the result of hybridization of DNA transferred to a membrane with one of the leading strand oligonucleotide probes. a, oligonucleotide 1; b, oligonucleotide, 3; c, oligonucleotide 5; d, oligonucleotide, 6. Sizes are in kb. The intensity of the 10.9 kb region was similar in each panel; the autoradiographs were overexposed to show clearly the ~1.2 kb species.

Fig. 3 shows the results for the leading strand probes. In addition to the expected species there are some minor bands of ≥ 2kb. While the origin of these bands is not known it has been established that they are not associated with the arrested forked molecule (K.S.Ahn and R.G. Wake, unpublished). They could reflect later events in the overall termination and segregation process i.e. after the first stage of fork arrest. Fig. 3 shows that the level of hybridization to the ~1.2 kb species falls off progressively and eventually to zero with the leading strand oligonucleotide probes 1, 3, 5 and 6. (The sequence corresponding to oligonucleotide 1 occurs in a region of the chromosome through which the majority, if not all, leading strands pass before being arrested; this will be substantiated in data presented below.) The significant drop in intensity of the ~1.2 kb species between the probings with oligonucleotides 1 and 3 (panels a,b) suggests that at least 50% of leading strands stop before IRII. The level detected with oligonucleotide 5 (panel c) is consistent with a small but significant fraction of the leading strands passing through IRII. But none pass through IRI (panel d). It should be noted that, as expected, the newly replicated strands hybridizing to the probes in Fig. 3 (also in Fig. 4 below) become longer as probe homology approaches the terminus. Hybridization of the same DNA with the lagging

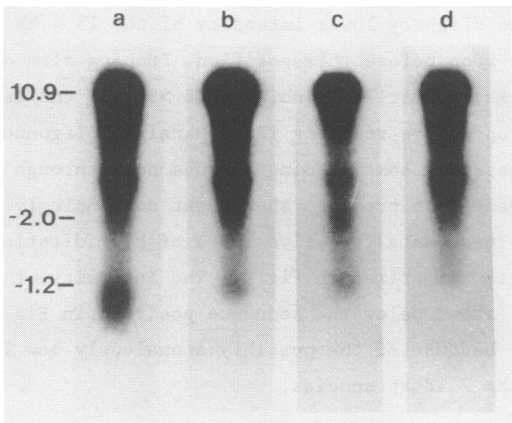

Fig. 4. Autoradiographs of *Bam*HI + *Eco*RI digests of DNA obtained from exponentially growing GSY1127 and fractionated by gel electrophoresis in alkali. Each panel was the result of hybridization of DNA transferred to a membrane with one of the lagging strand oligonucleotide probes. a, oligonucleotide 7; b, oligonucleotide 8; c, oligonucleotide 9; d, oligonucleotide 10. Sizes are in kb. The intensity of the 10.9 kb region was similar in each panel; the autoradiographs were overexposed to show clearly the ~1.2 kb species.

strand probes (oligonucleotides 7, 8, 9 and 10, Fig. 4) shows that the majority of the lagging strands also stop before IRII (panels a,b,c) with very few or none traversing IRII (panel d). The hybridization data for these experiments are summarized in semiquantitative form in Fig. 2.
An alternative approach to analysing the limits of the leading strands was used. This involved separation of the arrested forked molecule from the bulk of the DNA and probing of its single strand components with appropriate oligonucleotides. For this purpose *Bam*HI-cleaved DNA was used. In such digests the IRR (or *terC*) occurs in a fragment of 24.8 kb and the arms of the arrested fork are 15.4 kb (2). The forked DNA migrates much more slowly than linear 24.8 kb DNA. The region containing the forked DNA was cut from a neutral gel and the slice used directly to separate the fork into its single strands by electrophoresis in an alkaline gel. Several samples of the same DNA were processed together in single neutral and alkaline gels and finally transferred to a single nylon membrane. Individual lanes were cut from the membrane and hybridized separately with oligonucleotides 1,2,3,4 and 6 (Fig. 5). The approximately equal intensities of the 24.8 and 15.4 kb species after probing with oligonucleotide 1 (panel a) establishes that most, if not all, leading strands pass through this sequence in the chromosome. Most of these continue to proceed through the sequence corresponding to oligonucleotide 2 (panel b) but the slightly lower intensity of the 15.4 kb species suggests that some stop before this position. Probing with oligonucleotide 3 (panel c) confirms the earlier finding that >50% of the leading and lagging strands stop before reaching IRII. Panel d (oligonucleotide 4 probing) establishes that some leading strands pass through IRII, while panel e (oligonucleotide 6 probing) shows most convincingly that they do not traverse IRI. These data (relative level of hybridization to the 15.4 kb species) are also summarized in Fig. 2. The intensity of probing with oligonucleotide 4 (shown below the sequence position in Fig. 2) is indicated as ++(+) because of the possibly anomalously low level of hybridization to the 24.8 kb species.

DISCUSSION

The data for the leading and lagging strand limits presented here show that many of the clockwise replication forks (>50%) approaching the IRR stop before IRII, and none traverse IRI (Fig. 2). Also, a small but significant number of forks (at least the leading strands) pass through IRII. The data

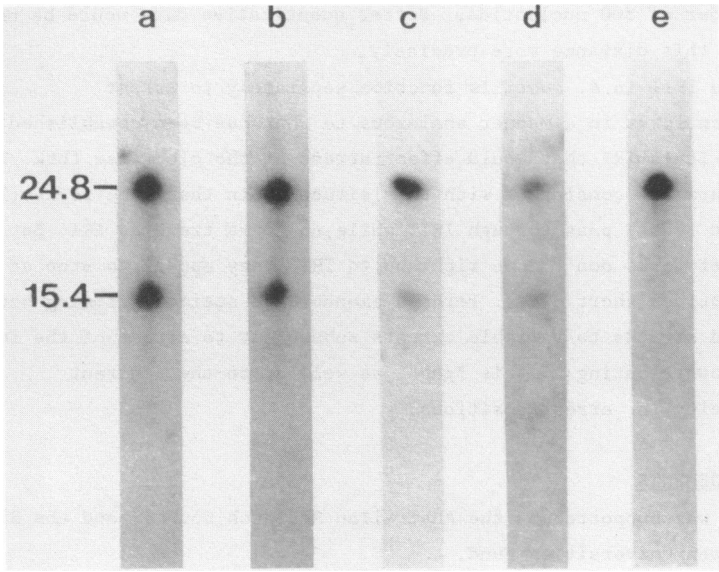

Fig. 5. Autoradiographs of single-strand DNA from the clockwise arrested fork isolated from a *Bam*HI digest of exponentially growing GSY1127 and then fractionated by gel electrophoresis in alkali. Each panel was the result of hybridization of DNA transferred to a membrane with one of the leading strand oligonucleotide probes. a, oligonucleotide 1; b, oligonucleotide 2; c, oligonucleotide 3; d. oligonucleotide 4; e, oligonucleotide 6. Sizes are in kb.

for the oligonucloeotide 5 probing supporting this last conclusion, which was obtained from analysis of the total *Bam*HI + *Eco*RI digest (Fig 3c) is possibly less reliable than that in Fig. 5d which relates to the isolated fork. This is because of the likely degradation of at least a small portion of the forks in the unfractionated *Bam*HI+ *Eco*RI digest. Such degradation would cause release of one of the ~1.2 kb double-stranded arms from the fork (15). If this released arm were the one containing the lagging strand the complement of this strand within the arm would hybridize to the same probe as *bona fide* leading strands and around the ~1.2 kb single-strand position. However, this material would not be expected to be longer than *bona fide* leading strands.

It is also clear that the clockwise forks stop over a range of distance in the sequence approaching the IRR, rather than at a site defined at the single nucleotide level. The data for the leading strands indicate that this distance is at least 100 nucleotides, from oligonucleotide 3 to oligonucleotide 5. The distance over which forks stop could be longer,

of the order of 300 nucleotides. Better quantitative data would be needed to define this distance more precisely.

If IRI and IRII in *B. subtilis* function separately to arrest replication forks in a manner analagous to what has been established for R6K (7,8) it is IRI that would effect arrest of the clockwise fork. The present data are consistent with this situation in that some forks (leading strands at least) pass through IRII while no forks traverse IRI. But the majority of forks don't come right up to IRI, they appear to stop at least 100 nucleotides short of it. Perhaps exonuclease action, trimming back the terminated strands to variable extents subsequent to arrest of the fork, is a factor contributing to this "gap", as well as to the apparent heterogeneity in arrest positions.

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