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Nucleotide sequence of *Escherichia coli* K-12 replication origin

(*Escherichia coli* chromosome/DNA cloning/autonomous replication/sequence homology)

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ABSTRACT **From subfragments of an *Eco*RI fragment (9 kilobase pairs) that contained the replication origin of the *Escherichia coli* chromosome and had been cloned as a recombinant with a nonreplicating DNA fragment coding for ampicillin resistance, small derivative plasmids were constructed. The smallest of these, pTSO151, contained a segment of 463 base pairs as the chromosomal component. Another plasmid, pSY314, constructed from *Bam*HI digests of the *Eco*RI fragment and mini-F(pMF21), contained a region of 422 base pairs identical with a corresponding region in pTSO151. We conclude that the replication origin of *E. coli* chromosome is located within this 422-base-pair segment. The nucleotide sequence of this segment is presented.**

Initiation of DNA replication seems to be determined by specific interaction of initiator protein(s) with the DNA replication origin, and in many respects this interaction appears to play a key role in the process of cellular division in bacteria (1–3). However, biochemical analyses of the initiation region have been hindered by the large bulk of chromosomal DNA. It is obvious that purification of the DNA initiation region is essential for such studies.

Recently, the replication origin of the *Escherichia coli* chromosome (*ori*) was successfully cloned as a recombinant (pSY211) of the origin-containing *Eco*RI fragment with a nonreplicating DNA fragment coding for ampicillin resistance (Amp^r) (4). However, the cloned *Eco*RI fragment still was 9 kilobase pairs long, which is too large for DNA sequencing of the *ori* region. For analysis of the sequence of origin, it was necessary to determine more precisely its location on the *Eco*RI fragment. Two approaches have been used for this purpose, based on the finding that *Bam*HI cleaved the *Eco*RI fragment into seven pieces, designated A to G in order of decreasing size (5) (see Fig. 1). One was the isolation of deletion derivatives from pSY211 by partial *Bam*HI digestion (4); the other was recloning of *Bam*HI fragments generated from the *Eco*RI fragment on mini-F(pMF21) (5). As a result, the replication origin was found to be contained within two *Bam*HI fragments, B and G, 2.2 kilobase pairs long (5).

In this paper, we show that the replication origin is contained within a short segment, 422 base pairs long, by cloning experiments with subfragments derived from the origin-containing *Eco*RI fragment. The nucleotide sequence of the defined region is also presented. Results complementary to our data will be described elsewhere (6, 7).

MATERIALS AND METHODS

***E. coli* Strains and Plasmids.** All strains used were derivatives of *E. coli* K-12. The strains used were C600 (F$^-$ *thr leu thi lac ton*A *sup*E), LC248 (HfrKL16 *rec*A), W3623*pol*A (F$^-$ *trp gal str pol*A11) (8), MM383 (F$^-$, *thy, rha, lac, str, pol*A12) (9), and A19 (Hfr *met rna*). Plasmids used were pSY211 (4), pSY221 (4), pSY314 (5), pYT10 (4), pMF21 (10), and pBR322 (11).

Enzymes. *Eco*RI, *Bam*HI, *Hin*dIII, *Hga* I, *Hae* III, *Hin*fI, *Hha* I, *Hap* II, *Bpa* I, *Bpa* II, and *Alu* I were prepared as described (12). *Sla* I, an isoscizamer of *Xho* I, was a gift from H. Takahashi. In this paper we use the name *Xho* I for comparison with other references. *Eco*RII was obtained from Bethesda Research Laboratories. DNA polymerase, DNA ligase, and polynucleotide kinase were prepared from T4 amN82-infected *E. coli* A-19 cells according to refs. 13–15, respectively.

Preparation of Plasmids. For preparation of origin-containing plasmids, cells harboring plasmids were grown in the presence of Amp (20 μg/ml) in a medium containing 10 g of peptone, 1 g of yeast extract, 10 g of glucose, 1 g of NH_4Cl, 3 g of NaCl, 0.1 g of Na_2SO_4, 0.08 g of $MgCl_2 \cdot 6H_2O$, 15.2 g of $Na_2HPO_4 \cdot 12H_2O$, and 3 g of KH_2PO_4 in 1 liter. Covalently closed circular plasmid DNA was prepared from cleared lysates (16) by using ethidium bromide/CsCl density gradient centrifugation (17). Plasmids containing the ColE1 replicon was prepared as described (18).

Preparation of Amp Fragment. The nonreplicating DNA fragment coding for Amp^r was prepared from pYT10 by *Eco*RI digestion as described (4).

Ligation of Restriction Fragments. Ligation of DNA fragments generated by the same restriction enzymes was carried out with T4 DNA ligase as described (19). Joining of restriction fragments generated by different enzymes was performed after the ends of the fragments were converted to fully base-paired ends as described (named "repair ligation") (19).

Gel Electrophoresis. Analysis of plasmids was carried out with 0.7 or 1% agarose gel columns (0.6 × 12 cm) in 40 mM Tris·acetate/20 mM sodium acetate/2 mM EDTA, pH 7.4. For analysis of restriction fragments, 5% polyacrylamide gel columns (0.6 × 12 cm) in Tris·borate/EDTA buffer (20) were used. The extraction of fragments from gel was performed as described (18).

Transformation. Cells were treated with $CaCl_2$ and transformed with purified plasmid DNA as described (21).

Sequence Determination. Restriction fragments were phosphorylated at the 5′-termini by the 5′-hydroxyl polynucleotide kinase reaction, and the two labeled ends were separated by secondary cleavage with different enzymes. The nucleotide sequence was determined by the method of Maxam and Gilbert (22).

Electron Microscopy. Plasmid DNA was prepared for electron microscopy by the basic-protein film technique of Kleinschmidt and Zahn (23) with some modification (24). Heteroduplex study of the plasmid DNAs was as described (25).

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Abbreviations: Amp^r, ampicillin resistant (resistance); Kan^r, kanamycin resistant (resistance); Tet^r, tetracycline resistant (resistance).

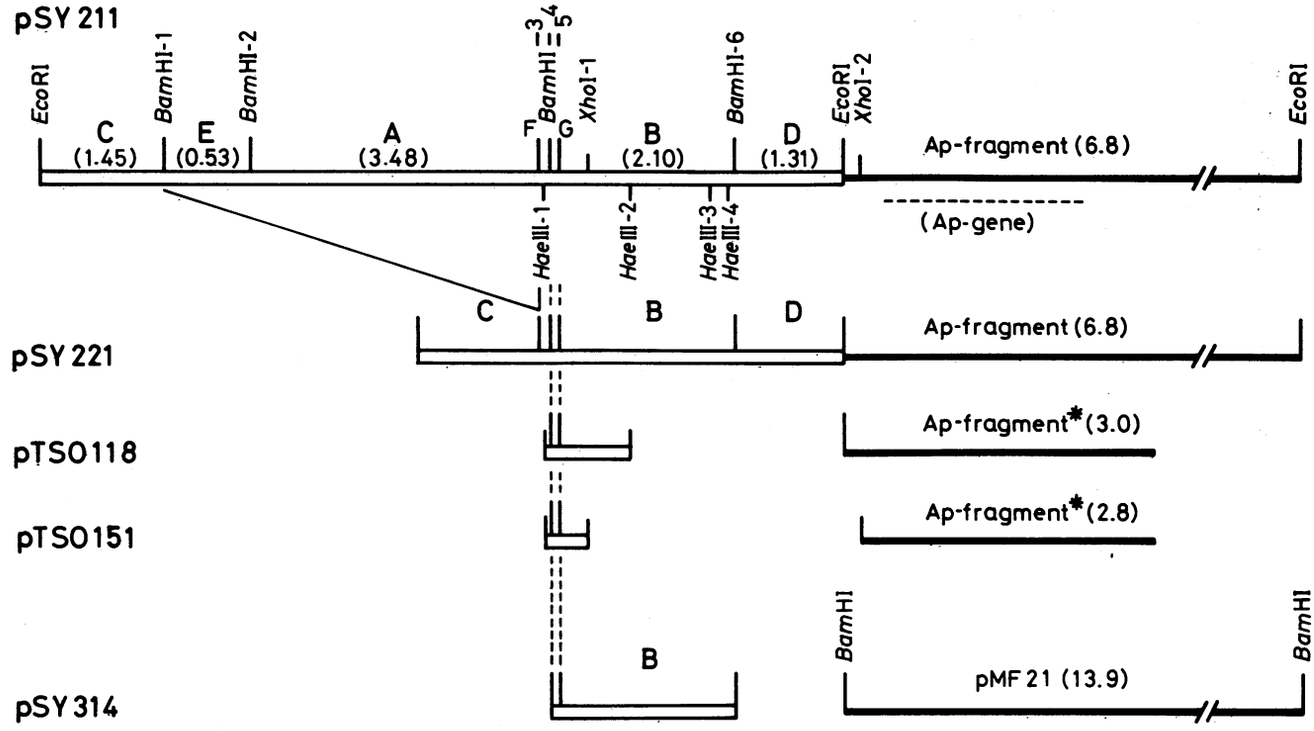

FIG. 1. Derivation and physical maps or *ori*-containing plasmids. Physical maps are shown in a linear form by opening the circular map at the junction between chromosomal and nonchromosomal DNAs. Open bars, chromosomal DNA; solid bars, nonchromosomal DNA. Seven *Bam*HI fragments from the *Eco*RI fragment of pSY211 were designated A to G in order of decreasing size and are indicated in the figure. Approximate fragment sizes (in kilobase pairs) are in parentheses. Sites of cleavage by *Bam*HI, *Hae* III, and *Xho* I are numbered from left to right. Only *Hae* III sites with the region of *Bam*HI fragments F, G, and B are indicated. Ap, Amp.

Containment. These experiments were conducted in a P2 facility.

RESULTS

Construction of Plasmids with Subfragments. As indicated on the cleavage map of pSY211 (Fig. 1), the *Bam*HI B plus G region can be divided into two major parts by *Hae* III: one from *Hae* III site 1 to site 2 and the other from *Hae* III site 2 to site 3 (corresponding to *Hae* III fragments D and E, respectively, in Fig. 3, lane c). Although the right end of *Bam*HI fragment B is not covered by these two *Hae* III fragments, the right one-third of *Bam*HI fragment B has been assigned to the *asn* gene from several lines of evidence (5). In order to determine which *Hae* III fragment carries *ori*, the two *Hae* III fragments were isolated from the *Hae* III digest of pSY221 and joined to the Amp^r fragment by repair ligation. By this procedure, *Eco*RI sites should be conserved at the junction between the *Hae* III and *Eco*RI fragments. The ligation products were transformed into LC248 (Hfr *rec*A), and Amp^r colonies were selected. A large number of Amp^r clones were isolated from the ligation products with *Hae* III fragment D but not with *Hae* III fragment E. Cells that had lost Amp^r rapidly segregated from the isolated Amp^r cells in the absence of Amp, as in the case of cells harboring the original pSY221. An exclusion process might be functioning during cell division.

Plasmid DNA was prepared from six transformants independently isolated and characterized by digestion with *Eco*RI, *Bam*HI, and *Xho* I. Two were recombinants of two *Hae* III fragment D and the Amp fragment (about 8.8 kilobase pairs), and the other two included three or more *Hae* III fragment D and the Amp fragment. This may have been due to the use of an excess of *Hae* III fragments for ligation. All these plasmids contained two *Eco*RI sites at the junction of the Amp *Hae* III fragments. The remaining two plasmids were smaller than the above plasmids (about 4 kilobase pairs) (Fig. 2, lane c), and *Bam*HI digestion yielded *Bam*HI fragment G. The physical map of one such plasmid, pTSO118, was constructed by restriction analysis (see Fig. 3, lanes d and e) and by heteroduplex analysis (data not shown) of pSY221 with pTSO118. pTSO118 was indeed a recombinant of *Hae* III fragment D with the Amp fragment, but the right half of the original Amp fragment was deleted as indicated in Fig. 1. Therefore, the *Eco*RI site was only formed at the junction of the right end of *Hae* III fragment D and the end near the *Xho* I site of the original Amp fragment. We interpret the result as showing that the Amp fragment

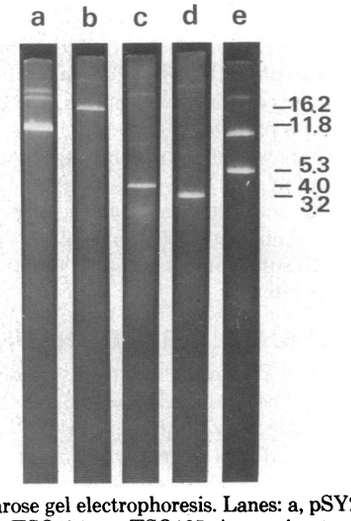

FIG. 2. Agarose gel electrophoresis. Lanes: a, pSY221; b, pSY314; c, pTSO118; d, pTSO151; e, pTSO125. Approximate sizes in kilobase pairs are indicated at the side. Bands of DNA in open circular form are present in lanes a and e.

generated by *Eco*RI from pYT10 contained such a short component probably due to the action of *Eco*RI* (26).

Isolation of Deletion Derivatives. pTSO118 contained two *Xho* I sites and two *Bam*HI sites (Fig. 1). Therefore, the regions flanked by each cleavage site can be removed by ligation of digests. In order to examine the dispensability of such regions, pTSO118 was digested by either *Xho* I or *Bam*HI, and ligation was carried out under conditions such that circular DNA was preferentially formed (27). The products were then transformed into LC248, and Amp^r colonies were selected. A large number of such colonies were formed from the ligation products of the *Xho* I digest but not of the *Bam*HI digest. As in the case of cells harboring pSY221 and pTSO118, Amp-sensitive cells segregated from Amp^r cells when cultured in the absence of Amp.

Plasmids were prepared from those six transformants that had been independently isolated and analyzed. All plasmids had the size estimated for the deletion product (3.2 kilobase pairs) (Fig. 2, lane d). The cleavage sites were examined on one such plasmid, pTSO151. The plasmid lacked the *Eco*RI site and contained a single *Xho* I site, as expected, so that double digestion with *Xho* I and *Bam*HI yielded three fragments, of which one was *Bam*HI fragment G (Fig. 3, lanes f and g). The above result therefore indicates that the region to the right of *Xho* I site 1 on *Hae* III fragment D is dispensable, but *Bam*HI fragment G is essential for autonomous replication. Another plasmid, pSY314, has been isolated from a kanamycin-resistant (Kan^r) clone of LC248 (Hfr *rec*A) transformed with the ligation products of the *Bam*HI digests of pSY221 and pMF21 (5). This plasmid contained only *Bam*HI fragments B and G as the chromosomal component (Fig. 3, lanes a and b). Therefore, we can conclude that *ori* is contained within the short region flanked by *Bam*HI site 4 and *Xho* I site 1 in Fig. 1.

Although we did not determine the exact number of copies of pTSO118 and pTSO151, the value estimated from the yields was about the same as that for the original pSY211. All these *ori*-containing plasmids were lost during cell division at a high frequency. Therefore, the properties of the original pSY211 appear to be maintained in these small derivative plasmids.

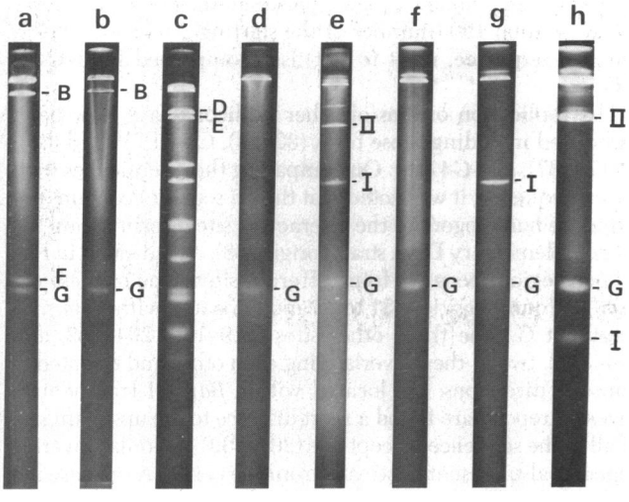

FIG. 3. Polyacrylamide gel electrophoresis of digests. Lanes: a, *Bam*HI digest of pSY221; b, *Bam*HI digest of pSY314; c, *Hae* III digest of pSY221; d, *Bam*HI digest of pTSO118; e, *Bam*HI/*Xho* I digest of pTSO118 [*Bam*HI fragment G and two fragments (bands I and II) flanked by *Bam*HI site 5 and *Xho* I site 1 and by *Xho* I site 1 and *Xho* I site 2 are indicated]; f, *Bam*HI digest of pTSO151; g, *Bam*HI/*Xho* I digest of pTSO151 [*Bam*HI fragment G and the fragment (band I) flanked by *Bam*HI site 5 to *Xho* I site 1 are indicated]; h, *Bam*HI digest of pTSO125 [*Bam*HI fragment G and two fragments (bands I and II) flanked by *Hae* III site 1 and *Bam*HI site 4 and by *Bam*HI site 5 and *Hae* III site 2 are indicated].

Cloning of the *ori*-Containing Fragment with pBR322. To examine the effect of increasing the number of *ori* copies in cells, *Hae* III fragment D containing *ori* was joined with the *Bam*HI digest of pBR322 by repair ligation which conserves *Bam*HI sites at the junction of the two fragments. The ligation products were transformed into C600, from which Amp^r tetracycline-sensitive (Tet^s) cells were selected. Plasmids were isolated from 16 independently isolated such transformants and examined by digestion with *Bam*HI. All the plasmids larger than pBR322 (four clones) were found to contain *Hae* III fragment D, indicating that the *ori* fragment can be cloned by the ColE1 replicon. These plasmids were stably maintained in C600 cells. Gel electrophoretic patterns of one of these plasmids (pTSO125) and its *Bam*HI digest are shown in Fig. 2, lane e, and Fig. 3, lane h, respectively.

We also constructed pBR322 recombinants of *Bam*HI fragment B (pTSO103), the fragment flanked by *Hae* III sites 2 and 3 (*Hae* III fragment E of pSY221) (pTSO116) and the fragment flanked by *Bam*HI site 5 and *Hae* III site 2 (pTSO109). When the transforming abilities of these plasmids into *pol*A cells were compared, pTSO125 gave a value more than 1000 times higher than the values for the three other recombinants, whereas the values of the four plasmids into *pol*A$^+$ cells (C600, LC248) were identical. In a thermosensitive *pol*A mutant (*pol*A 12) (9), replication of these four plasmids occurred normally at 31°C. At 41°C, however, only pTSO125 continued replication. The result provides evidence that the *ori*–ColE1 recombinant replicates in *pol*A cells by using the *ori* replicon and that *Bam*HI fragment G is indispensable for *ori* function.

Sequence of the Replication Origin. *Bam*HI fragments B and F and *Hae* III fragment D were prepared from pSY221, and the cleavage map was constructed with 13 different restriction enzymes. Based on the resulting cleavage map, the entire sequence from *Bam*HI fragment F to fragment B was determined by the method of Maxam and Gilbert (22). Part of *Bam*HI fragment B was sequenced by using the fragment amplified by pBR322 (pTSO103). The sequence was verified by duplicate analysis of the same fragment or by analysis of overlapping fragments until unambiguous results were obtained. In Fig. 4, the sequence from the left end of *Bam*HI fragment F to the middle of *Bam*HI fragment B (positions −105 to 1000) is shown. The sequence is numbered from left to right from the junction between *Bam*HI fragments F and G (*Bam*HI site 4 in Fig. 1). The upstream sequence is denoted by the minus sign. It was confirmed that pTSO151 contained positions −41 to 422 in the sequence of Fig. 4 from analysis of fragments generated from this plasmid by digestion with *Bam*HI and *Xho* I. pTSO118 covers the region from positions −41 to 974.

DISCUSSION

The origin of replication of the *E. coli* chromosome has now been localized within a segment of 422 base pairs (*ori* segment). The sequence in this segment is shown in the box in Fig. 4. The smallest plasmid constructed (pTSO151) contained only this *ori* segment and 41 additional base pairs as its chromosomal component. This provides strong evidence that all the sequence information required for autonomous replication is contained in the defined *ori* segment. We further showed that the region from positions 1 to 91, flanked by two *Bam*HI sites, is indispensable for *ori* function.

In another paper (6), the nucleotide sequence of a region of the *E. coli* chromosome containing *ori* is presented. Within the

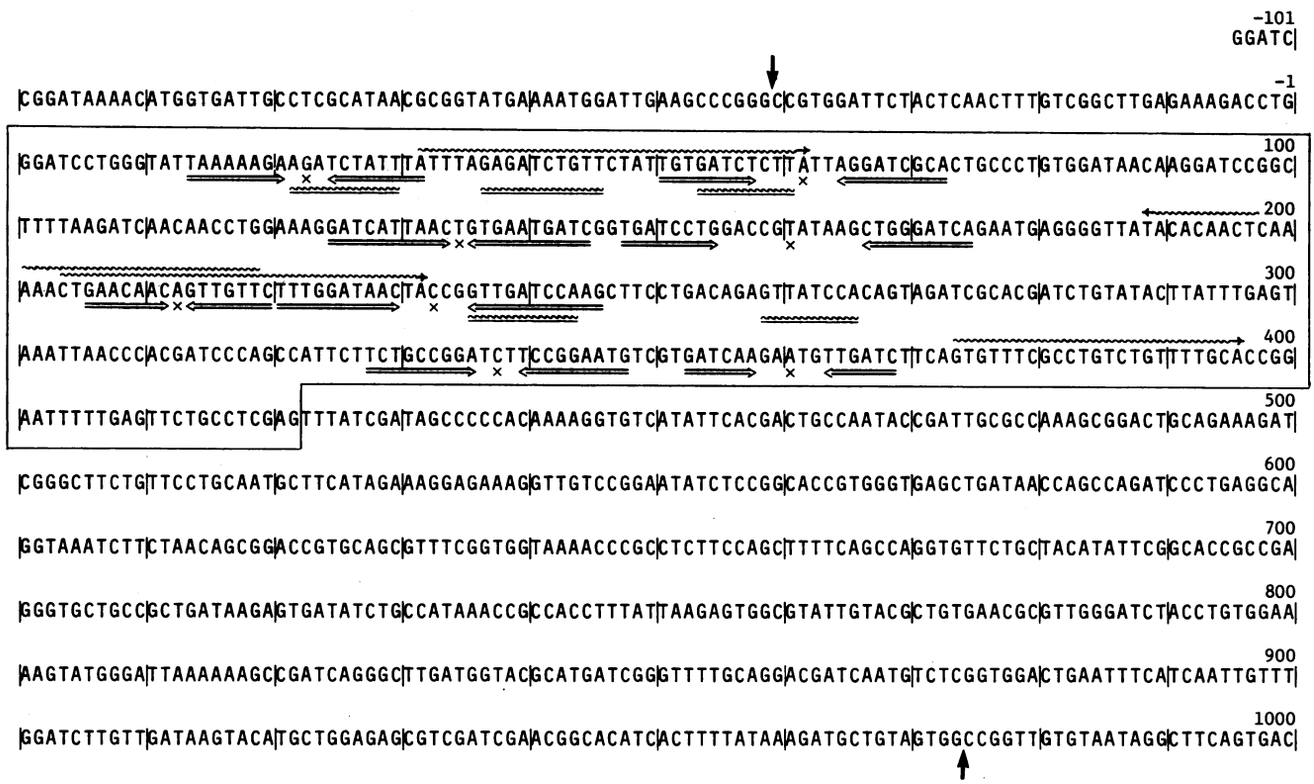

FIG. 4. Nucleotide sequence of the replication origin of *E. coli* K-12. Nucleotides are numbered from *Bam*HI site 4 (junction between *Bam*HI fragments F and G) in the left-to-right direction (5′ to 3′). The *ori* segment, defined as the overlapped region of pTSO151 and pSY314, is boxed. The region covered by *Hae* III fragment D(pTSO118) is indicated by arrows. Recognition sequences of some restriction endonucleases, indicated by nucleotide number at 5′ ends, are as follows: *Bam*HI, −105, 1, 92; *Hin*dIII, 244; *Xho* I, 417; *Bpa* I, 285; *Bpa* II, −70, 778; *Hph* I, −88, 146; *Hha* I, 476; *Hga* I, 930; *Bgl* II, 22, 38; *Hae* III, −43, 973; *Eco*RII, −4, 5, 116, 152, 668; *Alu* I, 165, 245, 572, 658; *Hap* II, −101, −46, 96, 233, 332, 341, 397, 546, 557, 975. Sequenced regions were both sides of *Bpa* I site 285, *Hin*dIII site 244, *Hha* I site 476, *Eco*RII site 668, *Bpa* II site 778, and *Hga* I site 930, the right side of *Bam*HI sites −105 and 92, *Alu* I sites 245 and 658, *Hap* II site 557, and *Hae* III sites −43 and 973, and the left side of *Bam*HI site 1 and *Hap* II sites 96 and 975. ⇒X⇐, Invert repeats; ⩙, direct repeats; ⇝, regions homologous to the *dna*G protein interaction site.

regions presented by the two groups, the sequences are identical. It should be noted that the respective *ori* fragments have been cloned by different selection methods from derivatives of *E. coli* K-12 [W3110 (4) and W208 (28)] that have been kept as separate cultures for 30 years in different laboratories.

A striking feature of the *ori* segment is that this region is particularly rich in various repeat sequences having the same or opposite direction, as indicated in Fig. 4. This strongly suggests that this region provides numerous interaction sites for protein(s).

Analysis of the coding capacity in the region covering the *ori* segment indicated that the regions from positions 361 to 750 and from positions 734 to 294 in the reverse direction can code for polypeptide chains of 130 and 147 amino acids, respectively. The former region is accompanied by a potential ribosome-binding site (29). However, the major part of the *ori* segment seems not to be translated into proteins, because termination codes frequently occur in all possible reading frames.

The initiation of replication of the *E. coli* chromosome has been shown to be sensitive to rifampicin (30), implying involvement of the RNA polymerase reaction in the initiation step. A promoter is defined by the presence of a sequence generally homologous to T-A-T-R-A-T-G (Pribnow sequence) in the region about 10 base pairs before the RNA initiation site (31). All of the promoters analyzed so far contain homologous sequences that differ from the Pribnow sequence by no more than two base pairs. Such sequences occur at 24 sites on the *ori* segment. It is known that many promoters contain a sequence homologous to T-G-T-T-G-A about 35 base pairs upstream from the RNA initiation site in addition to the Pribnow sequence (32). Among the 24 Pribnow type sequences in the *ori* segment, the one at position 190 (number of the starting nucleotide of the Pribnow sequence, right to left) is accompanied by T-G-T-T-G.

The replication origins of other replicons have now been sequenced including those for λ (33, 34), ColE1 (35), fd (36), φX174 (37), and G4 (38). On comparing these sequences with the *ori* sequence, it was noted that the *ori* segment contains the sequence homologous to the interaction site of primase on the G4 complementary DNA strand origin (38). As indicated in Fig. 4, such regions occur at four different sites. One of these occurred around positions 31 to 62 and is located within *Bam*HI fragment G. The three other sites (219–188, 204–232, and 374–396), two of them overlapping each other and oriented in opposite directions, are located within *Bam*HI fragment B. Inverted repeats are found a short distance to the upstream side of all of the sequences except one (204–232). A similar inverted repeat is also present upstream from the G4 RNA primase initiation site (38).

However, the sequence information on the *ori* segment provides only a few clues as to the mechanism involved in the initiation reaction. Also, it is not known whether the *ori* segment defined in this paper contains all the components for the replication origin of *E. coli*, which should include the sites for membrane attachment, bidirectional replication, and also regulatory regions of replication. The *ori* segment as well as plasmids containing this segment are now available for experimental use. This makes it possible to correlate the sequence of

the origin with its functional roles from both biochemical and genetic approaches.

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