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Nucleotide sequence of the *Salmonella typhimurium* origin of DNA replication

(*S. typhimurium ori* region/sequence homology/mismatch repair/secondary structure)

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ABSTRACT **Construction of deletion derivative plasmids and cloning of restriction fragments from plasmids containing the *Salmonella typhimurium* origin of replication (*ori*) were used to locate the functional origin to within a DNA fragment of 296 base pairs between the genes *uncB* and *asn*. The nucleotide sequence of the *S. typhimurium ori* region was determined and compared with the *Escherichia coli ori* sequence. In the 296-base pair fragment, 85.8% of the bases are conserved between the two species. A nearly equal number of transition and transversion type differences, with no insertions or deletions, occurs between the two bacterial origins, such that the relatively high percentage (adenine plus thymine) of 59.5% is conserved. The 296-base pair fragment contains 14 GATC sequences, all of which are conserved. The high frequency of occurrence of GATC, which is the site of methylation under control of the *dam* gene, may explain in part why the bacterial *ori* region appears to be so highly conserved. A large number of secondary structures are possible. One such structure, with a "cloverleaf," is favored by *ori* nucleotide sequence comparisons and leads to potential novel macromolecular interactions.**

Recently, plasmids have been isolated that contain the *Escherichia coli* (1–6) and *Salmonella typhimurium* (7) DNA replication origin, *ori*. The cloned *Eco*RI fragment containing the *S. typhimurium* origin of replication is 19.4 kilobase pairs long and includes functional *asn* and *uncB* genes (7), whereas the cloned *Eco*RI fragment containing the *E. coli* origin of replication is 9.0 kilobase pairs long and includes a functional *asn* gene but only part of the *unc* operon (2, 4, 5). Analysis of the *E. coli* origin plasmids has located *ori* to a 422-base pair (bp) region of the chromosome between the genes *uncB* and *asn* at 82 min on the *E. coli* genetic map. Here, we have determined more precisely the *S. typhimurium ori* location by isolation of deletion derivatives of *ori* containing plasmids and by recloning of *Bam*HI fragments in the plasmid pMK2004 (8), and show that the replication origin is contained within a DNA segment 296 bp long, between the *uncB* and *asn* genes.

The nucleotide sequence of the *E. coli ori* region has been determined independently by two groups (2, 3, 5, 6) with complete agreement, even though the *ori* fragments used by each group were derived from different *E. coli* K12 strains maintained as separate cultures for 30 years. Because of the divergence of *S. typhimurium* and *E. coli*, studies of the pattern of conserved and nonconserved regions of DNA sequences, for example, in the *trp* operon (9), have yielded sequence requirements necessary for regulatory gene activities. Here we report the DNA sequence of the *S. typhimurium ori* region and compare this sequence with the *E. coli ori* sequence. We propose one explanation for the high degree of conserved bases found in the bacterial *ori* regions and discuss one possible secondary structure that agrees with the sequence comparisons.

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MATERIALS AND METHODS

Bacterial Strains and Plasmids. The *E. coli* K12 strains used were ER *asnA31 asnB2 thi-1* F^+ (10), DF1323 *thy recA ΔtrpE5 thr leu thi lacY* F^- (7), D110 *thy endA polA1* F^- (11), and MM383 *thy lac rha rpsL polA12*(ts) F^- (12). The plasmids used were pMK2004 (8) and pJZ19 (7). Plasmid pJZ19 contains the *Pst* I fragment B of pJZ1 (7) inserted into the *Pst* I site of the *amp* gene of pMK2004 (Fig. 1). The plasmid pJZ1 consists of the *S. typhimurium Eco*RI fragment containing *asn*, *ori*, and *uncB* and the *Eco*RI fragment containing the *kan* gene.

Restriction Analysis and Plasmid Construction and Isolation. Enzymes, reaction conditions, DNA isolation, and DNA molecular weight determinations were as described (7). Transformation of *E. coli* was as described (13).

Labeling of DNA Fragments. DNA (60 μg) of pJZ19 was digested as outlined in Fig. 2. The 5′ ends were labeled by means of 20 units of phage T4 polynucleotide kinase (P-L Biochemicals) and 360 pmol of [γ-^{32}P]ATP (prepared by the S. I. T. Kennedy modification of that described in ref. 14; >6500 Ci/mmol; 1 Ci = 3.7×10^{10} Bq) in kinase buffer (100 mM Tris·HCl, pH 7.6/10 mM $MgCl_2$/5 mM dithiothreitol/0.1 mM spermidine/0.1 mM EDTA) after dephosphorylation of the DNA with bacterial alkaline phosphatase (Worthington). Incubations of these reactions were for 30 min at 37°C. After secondary restriction endonuclease cleavage, the DNA fragments were separated on 7% acrylamide gels and the labeled bands were eluted as described (15).

DNA Sequence Determination. Sequences were determined as described by Maxam and Gilbert (15). Dimethyl sulfate was used for Gua reactions; pyridinium formate (pH 2), for Gua + Ade reactions; hydrazine plus 5 M NaCl, for Cyt reactions; hydrazine, for Cyt + Thy reactions; and 1.2 M NaOH, for Ade > Cyt reactions. Each sample was divided into two portions, and the reactions were carried out for 5 and 30 min except for the pyridinium formate reactions, which were for 20 and 120 min. Times were decreased for fragments longer than 300 nucleotides. These portions were then recombined before cleavages. Cleavage reactions were carried out in 1.0 M redistilled piperidine for 30 min at 90°C. The samples were dried under decreased pressure, washed twice with water to remove residual piperidine, dissolved in 80% deionized formamide/50 mM Tris borate, pH 8.3/1.0 mM EDTA/0.1% xylene cyanol/0.1% bromphenol blue, denatured (90°C, 1 min, followed by quick-chilling in ice-water), and loaded onto gels for sequence determination. These polyacrylamide gels were 20% for cleavage products 1–30 ($19.4 \times 36 \times 0.04$ cm) and 8% for cleavage products 25–400 ($19.4 \times 36 \times 0.04$ cm for cleavage products 25–150 and $19.4 \times 85 \times 0.4$ cm for cleavage products ≥ 150). They were run in 8.3 M urea/100 mM Tris borate, pH 8.3/2 mM EDTA. Gels were run at 10–17 mA for desired times,

Abbreviations: bp, base pair(s); Km, kanamycin; *kan*, gene conferring kanamycin resistance; Ap, ampicillin; *amp*, gene conferring ampicillin resistance.

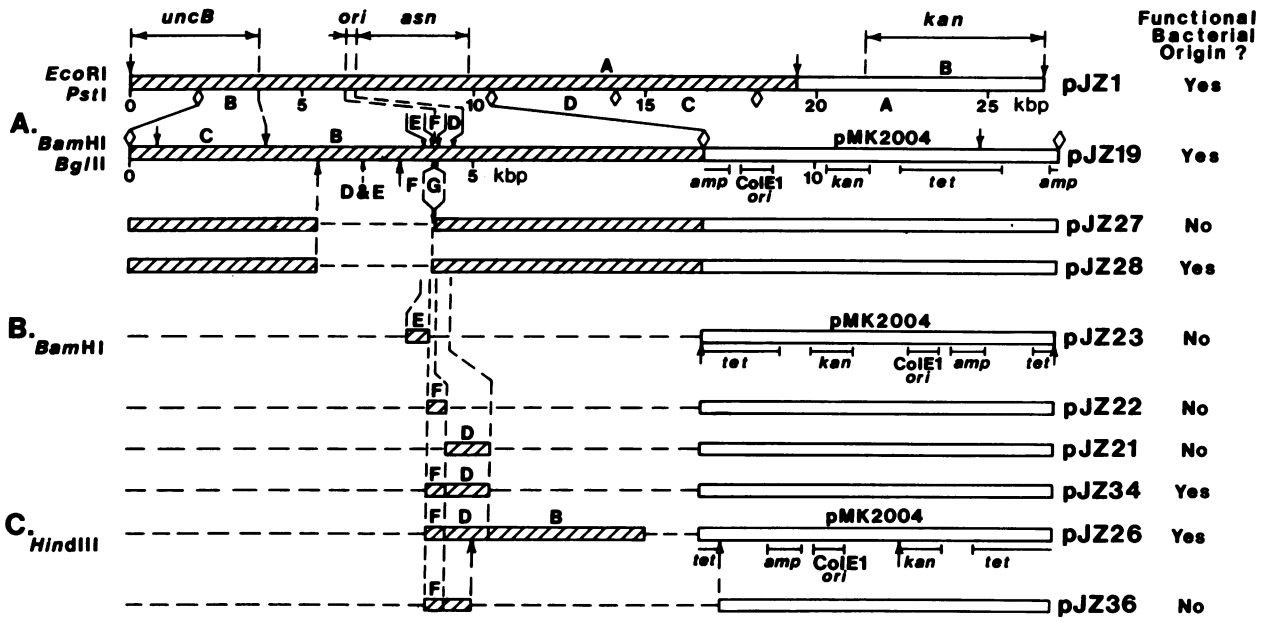

FIG. 1. *S. typhimurium ori* plasmids derived from pJZ1. (*A*) pJZ19 and *Bgl* II deletion derivative plasmids. (*B*) Plasmids containing pJZ19 *Bam*HI fragments. (*C*) pJZ26 and *Hin*dIII deletion derivative plasmid. Hatched regions designate *S. typhimurium* chromosomal DNA, and open regions indicate cloning vehicle DNA (*kan*-fragment for pJZ1, plasmid pMK2004 otherwise). Arrowheads, sites for restriction enzymes *Eco*RI, *Bam*HI, *Bgl* II, and *Hin*dIII shown on left; ◊, sites for the restriction enzyme *Pst* I; ---, deleted DNA. For clarity of presentation, *Bam*HI fragments D, E, and F in plasmids pJZ23, pJZ22, pJZ21, pJZ34, pJZ26, and pJZ36 are shown enlarged 3-fold. kbp, kilobase pairs.

and the DNA bands were visualized on Kodak XR-2 x-ray film with Ilford fast tungstate intensifying screens at −70°C.

RESULTS

Bgl II Deletion Derivative Plasmids. Construction of the plasmid pJZ19 by insertion of pJZ1 *Pst* I fragment B (Fig. 1) into the *Pst* I site of pMK2004 (8) has been described (7). To determine if any nucleotides between positions 1 and 37 (Fig. 4) are required for origin function, pJZ19 DNA was digested with *Bgl* II, self-ligated, and used to transform *E. coli* strain MM383 *polA12*(ts), selecting for kanamycin (Km)-resistant clones at 24°C. Plasmids containing only ColE1 origins—e.g. pMK2004—cannot replicate in *E. coli polA* polymerization mutants (16). Thus, pMK2004 can be maintained in *E. coli* MM383, which contains a thermosensitive *polA* polymerization mutation (*polA12*), at 24°C but not at 43°C (data not shown). Km-resistant clones obtained at 24°C that could also form Km-resistant clones at 43°C all contained plasmids deleted for the *Bgl* II fragments D, E, and F, but not for fragment G (e.g., pJZ28, Fig. 1*A*). These plasmids are also stably maintained in *E. coli* strain D110 *polA1*. In contrast, those Km-resistant clones obtained at 24°C that could not form clones at 43°C all contained plasmids deleted for *Bgl* II fragments D, E, F, and G (e.g., pJZ27, Fig. 1*A*), and these plasmids could not transform *E. coli* D110 to Km resistance. Thus, the base pairs between positions 1 and 21 (Fig. 4) are not required, but *Bgl* II fragment G is required, for a functional bacterial origin. We have shown (7) that pJZ1 *Bam*HI fragment E (Fig. 1) is not required for origin function. Further, the pJZ1 *Bgl* II fragment G was deleted from plasmid pJZ34 (Fig. 1*B*; see below) via digestion of isolated pJZ34 with *Bgl* II, self-ligation, and transformation of *E. coli* DF1323, selecting for Km-resistant, ampicillin (Ap)-resistant colonies. Plasmid DNA isolated from four such clones was shown to be deleted for pJZ1 *Bgl* II fragment G by restriction analysis and did not contain a functional bacterial origin when tested by transformation of *E. coli polA* mutants. This plasmid, pJZ37, demonstrates that the pJZ1 *Bgl* II fragment G is required for a functional bacterial origin even when nucleotides 1–21 (Fig. 4) are present.

Cloning of pJZ19 *Bam*HI Fragments. *E. coli* origin plasmids containing a deletion to the right of the *Xho* I site near *E. coli* position 422 (Fig. 4) still contain a functional bacterial origin (2, 3, 5, 6). A requirement for nucleotides to the right of the *Bam*HI site near position 317 (Fig. 4) for a functional *S. typhimurium* origin was tested by cloning of pJZ19 *Bam*HI fragments into the *Bam*HI site in pMK2004. A *Bam*HI digest

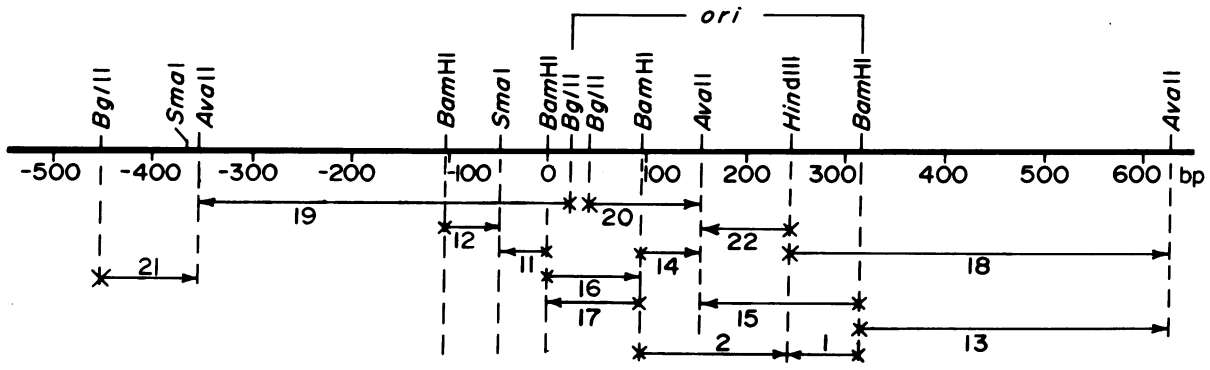

FIG. 2. Strategy for Maxam–Gilbert sequence determination of *S. typhimurium ori* region. X, ^{32}P-labeled end of each fragment; arrowhead, direction of sequence determination. The numbers given to individually sequenced fragments are those from experimental data.

of pJZ19 was ligated with a *Bam*HI digest of pMK2004, and the mixture was used to transform *E. coli* MM383 at 24°C or *E. coli* D110 at 37°C, selecting for Km-resistant, tetracycline-sensitive clones. All of the plasmids isolated from *E. coli* D110 clones, or from *E. coli* MM383 clones that could also form Km-resistant clones at 43°C, contained at least pJZ1 *Bam*HI fragments D and F, the smallest plasmid being pJZ34 (Fig. 1*B*). Km-resistant *E. coli* MM383 clones obtained at 24°C that could not form Km-resistant clones at 43°C lacked pJZ1 *Bam*HI fragment D or F or both (e.g., pJZ21, pJZ22, and pJZ23; Fig. 1*B*). Thus, nucleotides to the right of the *Bam*HI site near position 317 may be deleted without destroying origin function.

HindIII Deletion Derivative Plasmids of pJZ26. To determine if nucleotides to the right of the *Hin*dIII site near position 249 are required for a functional *S. typhimurium* origin, plasmid pJZ26 (Fig. 1*C*) was digested with *Hin*dIII, self-ligated, and used to transform *E. coli* strain MM383 *polA12*, selecting for Km-resistant, Ap-resistant clones at 24°C. Such clones contained only plasmids of the type pJZ36 (Fig. 1*C*), and these clones were unable to form Km-resistant clones at 43°C. These plasmids were also unable to transform *E. coli* D110 to Km resistance. Thus, nucleotides to the right of the *Hin*dIII site near position 249 are required for a functional bacterial origin, and we conclude that the functional *S. typhimurium ori* region is contained within the 296-bp region between positions 22 and 317.

Nucleotide Sequence Determination of the *ori* Region. The chemical method of Maxam and Gilbert (15) was used to determine the nucleotide sequence of the *S. typhimurium ori* region, using pJZ19 DNA and the strategy indicated in Fig. 2. Two independent approaches permitted determination of the sequence of each nucleotide at least twice and through each restriction site used. In the first approach, the 5′-ends of a *Bam*HI digest of pJZ19 were labeled with ^{32}P. The labeled fragments were then digested with *Ava* II (fragments 13, 14, and 15), with *Sma* I (fragments 11 and 12), with *Hin*dIII (fragments 1 and 2), or they were melted to separate strands (fragments 16 and 17). In the second approach, *Bgl* II (fragments 19, 20, and 21) and *Hin*dIII (fragments 18 and 22) ^{32}P-labeled fragments were digested with *Ava* II. Fragments were purified by using agarose or acrylamide gel electrophoresis, and individual fragments were subjected to five of the Maxam–Gilbert sets of chemical reactions. Data for fragments 14 (63 bp) and 15 (157 bp) are shown in Fig. 3; these two fragments comprise 220 bp of the minimal 296-bp *ori* region. The resulting nucleotide sequence of the *S. typhimurium ori* region, with comparison to that of *E. coli*, is shown in Fig. 4.

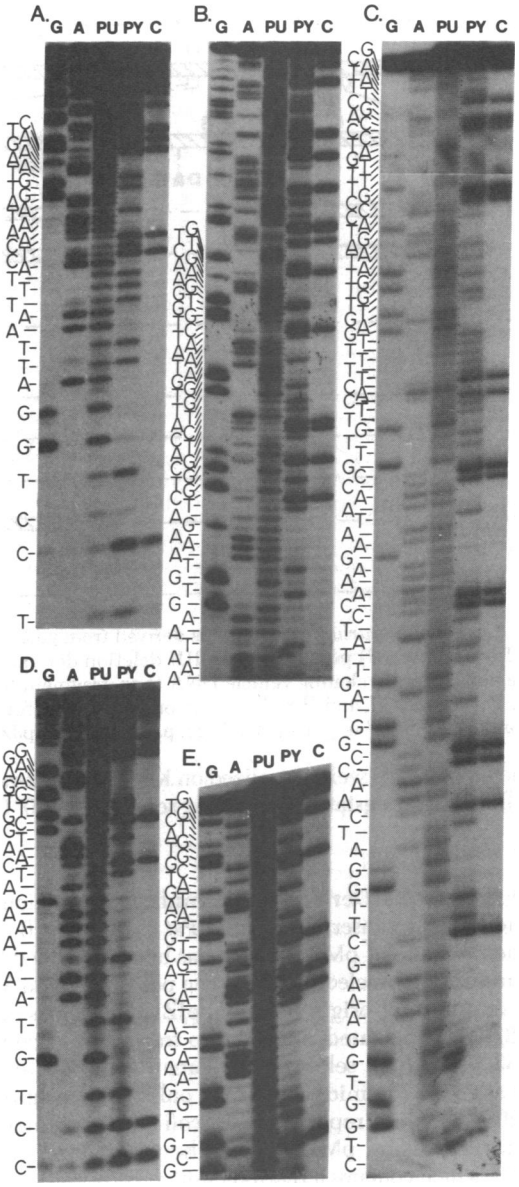

FIG. 3. Representative urea/acrylamide gel electrophoresis "sequence ladders." (*A*) Fragment 15, 20% gel, nucleotides 314–280. (*B*) Fragment 15, short 8% gel, nucleotides 296–243. (*C*) Fragment 15, long 8% gel, nucleotides 257–159. (*D*) Fragment 14, 20% gel, nucleotides 96–125. (*E*) Fragment 14, short 8% gel, nucleotides 114–155. PU, cleavage at purines; PY, cleavage at pyrimidines.

DISCUSSION

By constructing deletion derivative plasmids and cloning *Bam*HI fragments, the *S. typhimurium* origin for DNA replication (*ori* region) has been localized to within a 296-bp region defined by *Bgl* II and *Bam*HI restriction sites. Because the pJZ1 *Bgl* II fragment G and an unknown number of nucleotides to the right of the *Hin*dIII site near position 249 are required (Fig. 1), this minimal origin is found between positions 22–37 and 250–317, with a resulting size of 213–296 bp. This size may be compared with the *E. coli* 422-bp *ori* region defined by the *Bam*HI site near position 1 and the *Xho* I site near position 422 (2, 3, 5, 6). More recently, Hirota and coworkers (17) have shown via site-specific deletion and insertion analysis that the minimal *E. coli ori* region is localized between positions 23–34 and 266–267, with a resulting size of 232–245 bp, in agreement with our *S. typhimurium ori* results. Smaller but still functional origins may also result upon deletion of bp within the 296-bp *S. typhimurium ori* region. We also note that the criterion used for a functional bacterial origin is simply that its presence converts a DNA molecule into a replicon that can be stably inherited and can lead to formation of bacterial colonies on selective media.

Even though *E. coli* and *S. typhimurium* are both members of the Enterobacteriaceae with similar genetic maps, they are not closely related. For example, DNA·DNA hybridization studies indicate only a 46% homology overall (18), and recombination between DNA molecules of the two species leads primarily to tandem duplications (19). In *E. coli rec*$^+$ recipients, we have not observed any integration of the *S. typhimurium* origin plasmids into the *E. coli* bacterial chromosome, in contrast to *E. coli* origin plasmids (1, 5). A direct comparison, then, of the nucleotide sequence of the *ori* regions of the two species would provide information on (*i*) the need for conservation of this region, (*ii*) which nucleotides can be changed and still yield a bacterial origin functional in *E. coli* (in this sense, *S. typhimurium* could be viewed as equivalent to a "multiply

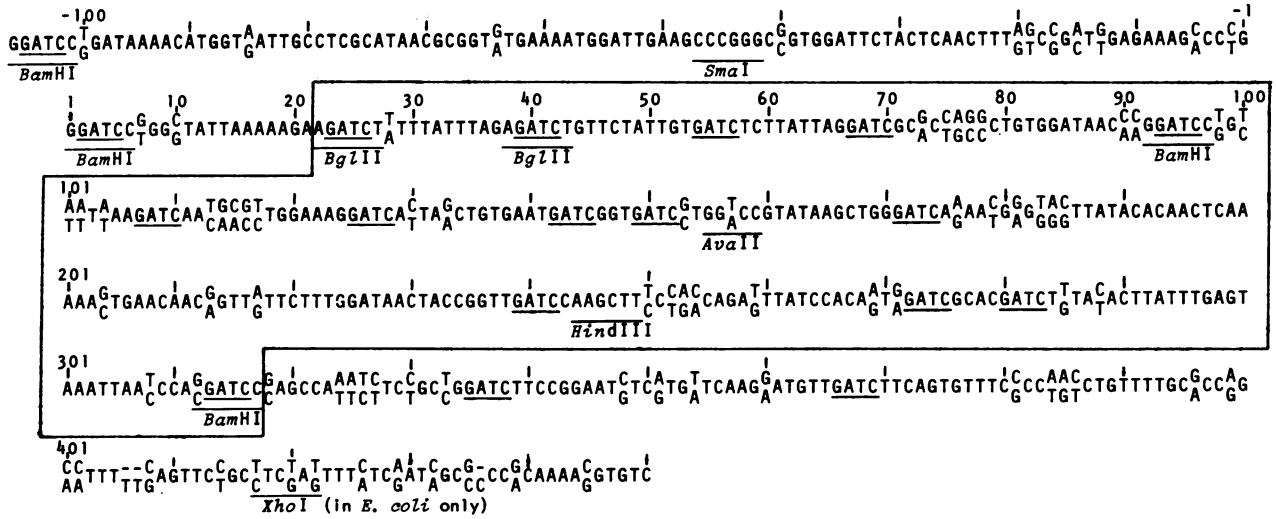

FIG. 4. Nucleotide sequence of *S. typhimurium ori* region. Nucleotides in the top line of each row are *S. typhimurium*, in the bottom line they are *E. coli*, and in the middle they are identical in the two species. The numbering is that used for the *E. coli ori* region (2, 3). The 296-bp region required for a functional *S. typhimurium* origin is shown within the box. The restriction sites are those of *S. typhimurium* except for the *Xho* I site. The upper left end is the 5′ end.

mutated *E. coli*"), and (*iii*) provide a basis for prediction regarding binding sites and other initiation functions of individual or groups of nucleotides.

Comparison of the nucleotide sequences of the two bacterial origins (Fig. 4) shows that the predominant feature is the very high degree of homology between the two origins: 85.8% in the 296-bp *ori* region, 89.3% in the region −106 to 21 (5′ side), and 77.3% in the region 318 to 450 (3′ side). Within the 296-bp *ori* region, 42 nucleotide differences occur, with no insertions or deletions, of which 22 are transitions and 20 are transversions. This region is 59.5% Ade + Thy, whereas both the 5′ and 3′ sides are close to 50% Ade + Thy in both species. A clustering of the nucleotide differences is evident within the *ori* region, with up to 43 bp of complete homology. This clustering is less evident to the 3′-side, with nucleotide differences often occuring every third nucleotide.

A striking feature of the sequence is the presence of 14 GATC sequences within the 296-bp *ori* region, plus 4 additional ones just outside the required *ori* region (Fig. 4). Only one such sequence per 256 bp is expected at random. As a specific hypothesis, the presence of the GATC sequences may in part account for the apparent high degree of conservation of the *ori* region in bacterial species. The *E. coli* K12 *dam* methylase recognizes specifically the GATC site, converting the Ade bases into 6-methylaminopurine (20). Further, repair of mismatched bases is deficient in *dam*⁻ mutants (21), and Wagner and Meselson (22) have proposed that undermethylation of newly replicated DNA could provide the basis for discrimination between the "correct" parental strand and the "error-containing" newly synthesized strand. If the interaction of mismatch repair enzymes with GATC sites tended to localize the mismatch repair enzymes to regions containing GATC sites,

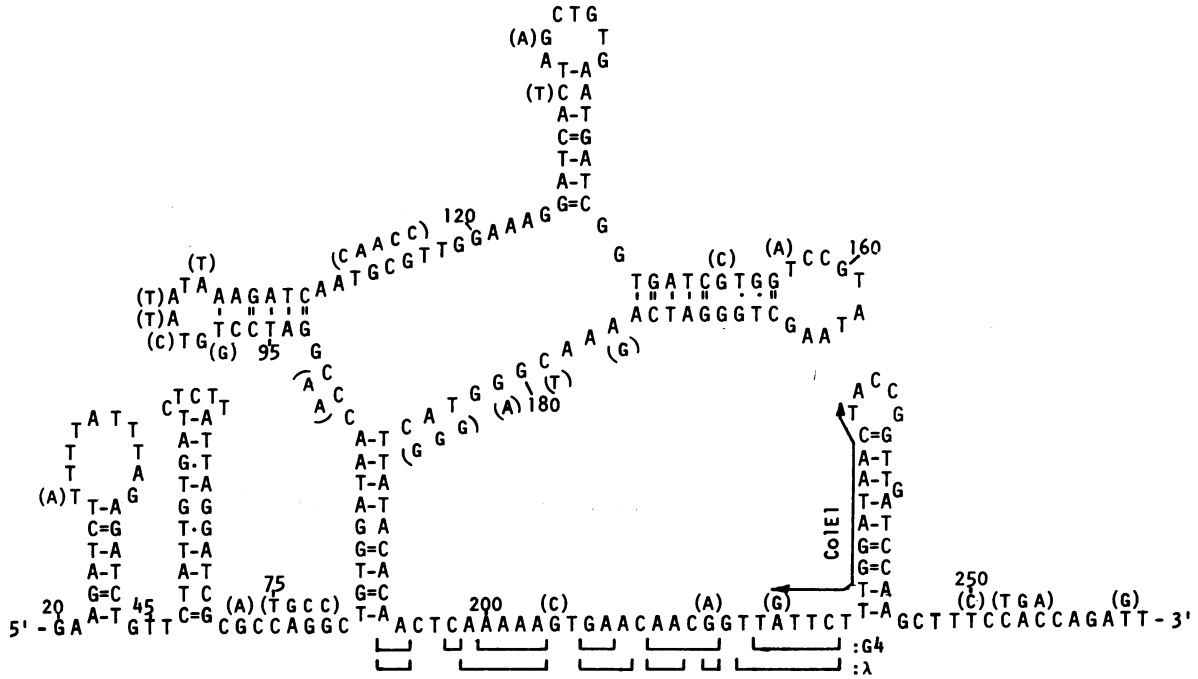

FIG. 5. A possible "cloverleaf" secondary structure for bacterial *ori* region. The sequence is that of *S. typhimurium*; *E. coli* nucleotides that differ are in parentheses. Nucleotides identical between the *E. coli ori* region and those of the bacteriophage G4 and λ *ori* regions are shown in brackets. Arrow, region of sequence homology between the ColE1 and bacterial *ori* regions.

then errors introduced during replication into a region rich in GATC sites would have a greater chance of being repaired by the mismatch repair system, leading to greater conservation of the nucleotide sequence.

The bacterial *ori* region contains an abundance of possible secondary structures, as seen via inverted repeats or direct repeats (2, 3, 5, 6) and via the computer diagram of Messer *et al.* (6). A specific structure that is favored by *ori* sequence comparisons and that leads to novel predictions is shown in Fig. 5. Region 194–221, which shows extensive homology with both the complementary strand *ori* of phage G4 (23, 24) and phage λ *ori* (25, 26), and region 222–245 of the proposed hairpin to the 3′ side discussed by others (2, 3, 5, 6) show complete homology between the two bacterial origins except for three differences, one of which shows no homology with either G4 or λ origin (position 204). This region also includes a 15-bp sequence that is identical to one present in the plasmid ColE1 origin (27), except for position 222 (Fig. 5, arrow). Two additional hairpin structures are proposed, positions 47–71 and positions 22–43. This latter hairpin is formed from the two *Bgl* II sites (see Fig. 4). All hydrogen-bonded nucleotides in these hairpins are conserved between the two bacterial *ori* regions.

The most striking proposed structure is the skewed cloverleaf structure in region 80–194. Such structures were proposed by Hobom and coworkers (26, 28) as possible structures in each of the four lambdoid phage *ori* regions whose nucleotide sequence was determined, and are in the same location in the *ori* region relative to the sequence homology region (positions 194–221). Within the four intrastrand hydrogen-bonded arms of the cloverleaf structure, all of the nucleotides are conserved between the two bacterial origins except for three. Thus, the "clusters" of nucleotide differences between *E. coli* and *S. typhimurium* are not found in the proposed intrastrand hydrogen-bonded regions. Rather, they are found in the interstrand hydrogen-bonded regions (e.g., regions 73–78 and 250–259), in the open regions of the hairpins and cloverleaf arms (e.g., region 100–104), and in the middle regions of the cloverleaf (e.g., regions 113–117 and 176–185). Although there are only 42 differences between the two bacterial *ori* nucleotide sequences, even these few differences often appear to leave unchanged what may be the important properties. As two examples, the open regions of the hairpin at positions 22–43 and of the cloverleaf arm at positions 93–110 are rich in adenines and thymines, and the middle regions of the cloverleaf in this strand are high in purines; these features are preserved in both bacterial sequences. The same is true concerning a similar cloverleaf structure for the opposite strand. The possibility of such extensive secondary structure may explain in part why origin regions appear to be large compared with regulatory regions such as operator–promoter regions.

Consideration of such structures introduces novel possibilities for macromolecular interactions in the *ori* region. As one specific possibility, the G-A-T-A-A-C-C sequence in this strand (Fig. 5) beginning at position 84 and the T-A-T-A-A-G-T sequence in the opposite strand beginning at position 191, are potential "Pribnow boxes" (29) with four of seven and five of seven bases correct, respectively, and are located in comparable positions of their respective cloverleaf structures. The correct bases are those that should be correct from hydrogen-bonding and sequence comparison considerations. If these are tight-binding sites for RNA polymerase transcription events, although no binding sites for RNA polymerase within the *E. coli ori* region have yet been detected (6), transcription would initiate 90–95 nucleotides away from each Pribnow box rather than six to seven bases away and would proceed *toward* the Pribnow boxes rather than *away* from them.

Further, such a hypothesis would predict that a single tightly bound RNA polymerase molecule might protect a nucleotide sequence at about position 84 as well as a nucleotide sequence at about position 190 from deoxyribonuclease digestion, thus providing evidence for a direct spatial interaction of these sequences. Such potential macromolecular interactions would arise only from such secondary structures as the cloverleaf described above.

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