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DNA sequence of the *E. coli gyrB* gene: application of a new sequencing strategy

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ABSTRACT

We have determined the sequence of the E. coli gyrB gene, using a new sequencing approach in which transposition from a mini-Mu plasmid into the DNA provides random start points for dideoxynucleotide sequence analysis. The gyrB sequence corresponds to a protein 804 amino acids long; a previously isolated protein fragment with partial enzymatic activity has been identified as the C-terminal half-molecule. A plausible terminator of gyrB transcription is located just beyond the structural gene.

INTRODUCTION

DNA gyrase from E. coli is made up of two subunits, A and B, with molecular weights of about 100,000 and 90,000, respectively. The corresponding structural genes have been identified, originally as loci of mutations to drug-resistance: one class of mutations in gyrA is responsible for resistance to quinolone compounds (e.g., nalidixic acid, oxolinic acid, and norfloxacin), while a class of mutations in gyrB confers resistance to the coumarin antibiotics novobiocin, coumermycin A_1, and clorobiocin. The use of these inhibitors has helped to show that the A subunit is responsible for the DNA breakage-rejoining activity of DNA gyrase, and that the B subunit carries out ATP hydrolysis. The DNA-supercoiling and DNA-relaxing activities require the presence of both subunits; the B subunit is apparently needed not only for the energy coupling used in the supercoiling reaction but even for the less demanding relaxation reaction. The isolation of a fragment of the Gyr B protein which was able to confer a DNA-relaxing activity on the Gyr A protein supported the idea that there are distinct functional domains of the B subunit; this complex was no longer active in DNA supercoiling or ATP hydrolysis. (For reviews incorporating the information cited above, see (1, 2)).

As a first step in more detailed studies of the functional organization of the gyrB gene, we have now determined the DNA sequence of the gene and its

surrounding region. The sequence determination was carried out with a novel sequencing strategy which is described in some detail below.

Transposon-Directed Sequencing

When a long DNA sequence is being determined, the ordering of individual sequenced segments becomes a major problem. It is possible to make a detailed restriction map in order to locate each segment, but this procedure quickly grows laborious as the length of the DNA increases. Random sequencing schemes, in which unselected subfragments are sequenced and the overlapping segments later assembled into a continuous sequence, have considerable advantages especially now that computer programs are available to aid in assembling the sequence (3). The fact that much of the sequence is determined several times over by the random sequencing method serves to lessen the frequency of accidental mistakes. However, with a totally random method, the number of sequencing reactions and the cumulative length of the sequence that has to be determined can become excessive, for a number of reasons. In the original random method, restriction enzymes or sonication were used to generate short fragments of DNA to be subcloned into an M13 sequencing vector (4,5,6). In this method, information about the location of each fragment in the original piece of DNA is lost in the subcloning process. An alternative method that uses subclones of limited exonucleolytic digestion products of a fragment to be sequenced has an advantage in this regard, since the location of the sequence derived from each subclone can be learned from the size of the fragment (7).

All the methods described so far use *in vitro* cloning for the generation of a library of subclones. However, one can also use an *in vivo* process to generate the necessary library. The method described below uses a new approach to the generation of random start points for DNA synthesis, starting at a universal primer oligonucleotide, in the chain-terminating sequencing reactions of Sanger *et al*. (4, 8). If a transposon is allowed to insert at many different points in the cloned piece of DNA to be sequenced, a primer homologous to the end of the transposon permits sequencing of a stretch of several hundred bases of DNA adjacent to each point of insertion. If the inserts are fairly randomly distributed, as is typical of transpositional recombination mediated by a certain class of transposing elements, a large enough number of inserts will cover the whole region with any desired degree of overlap, allowing the entire sequence to be assembled.

Key factors in making the method practical are that the transposon must:

1. transpose at high frequency, yet the product transposant must maintain a stable structure;

2. insert itself almost randomly into the DNA sequence;

3. be a DNA fragment short enough to allow easy processing of the clones with inserts;

4. carry an easily selectable marker to allow convenient selection of the transposants.

All these conditions can be met with suitable derivatives of bacteriophage Mu. Mu transposes at a higher frequency than any other known transposon, and inserts with little sequence specificity into other DNA. Only the two Mu end sequences are needed to form a mobile DNA element, called mini-Mu, which can transpose if the Mu A and B proteins are supplied along with other factors present in the E. coli host cells. On the other hand, the sequence of DNA containing mini-Mu is stable in the absence of the phage coded functions (9,10). Plasmids containing mini-Mu transposons have been constructed, in which a drug-resistance gene is flanked by the two Mu-end sequences, as shown in Fig. 1.

If one co-infects an E. coli strain carrying the mini-Mu plasmid with an M13 phage vector containing a cloned piece of DNA to be sequenced and with phage Mu (as donor of the Mu A and B functions), the progeny will include transducing M13 phages in which the drug-resistant mini-Mu transposon is inserted into the cloned segment. (Alternatively, the transposition functions of Mu can be supplied by inducing cloned genes coding for the necessary proteins, as described below.) These phages are isolated by transducing a second E. coli strain to drug-resistance. The viral DNA of each transducing phage can then be used directly, in combination with a synthesized oligonucleotide primer complementary to the Mu-end sequence, for sequencing by the chain terminator method (8).

MATERIALS AND METHODS

Bacterial Strains, Phages, and Plasmids

E. coli strains used in this study are all derivatives of JM103; Δ*lacpro*, *supE*, *thi*, *strA*, *sbcB15*, *endA*, *hspR4*, F'*traD36*, *proAB*, *lacI*^{Q}, *lacZ*ΔM15 (6) or JM105; Δ*lacproAB*, *thi*, *rpsL*, *endA*, *sbcB15*, *hspR4*, F'*traD36*, *proAB*, *lacI*^{Q}, *lacZ*ΔM15 (11). AT32 is JM103 Mu^{R}; AT33 is AT32 (λcI857::Tn9); AT40 is JM103 (pAT38); AT42 is AT33 cured of lambda but still retaining Tn9. Plasmid pAT38 is described below. A parallel set of strains in the JM105 background was also constructed and used for some experiments. AT44 is SG13508 (F'*lacI*^{Q}, *lacZ*::Tn5 *pro*$^{+}$); it was made from SG13508, which is a *gal*$^{+}$ derivative of N5340; F^{-}*his*, *ilv*, *proC*, *galE*::Tn10, Δ(*chl-pgl*)8, (λΔ*BamcI857*Δ*H1*),

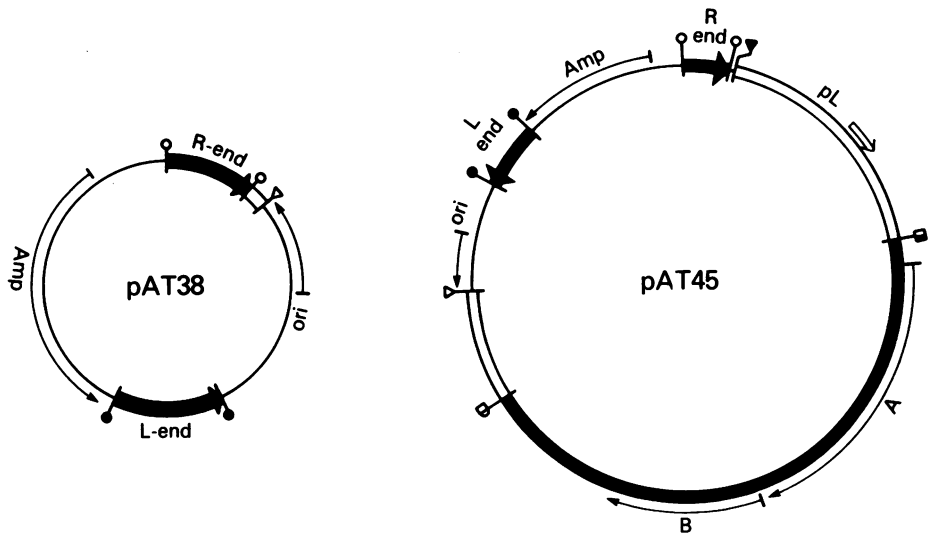


Fig. 1. Structures of pAT38 and pAT45. See text for construction of the plasmids. The sources of the DNA fragments are: pBR322, thin lines; phage Mu, thick lines; phage lambda, double lines. Arrows outside of each circle indicate the positions of genes, the origin and direction of replication of pBR322 (the tip of the arrow is the origin), and the lambda pL promoter as marked. Restriction sites indicated are: EcoRI, open circles; BamHI, open triangles: XhoI, filled circles; HindIII, filled triangle. A fused junction between an HpaI site and a BalI site is denoted by a half-filled square, and a fused junction between an HpaI site and an EcoRI site by an open half square and half circle.

Δ(cro-bio-uvrB), strA, thi (12), and GC2438. GC2438 is Δlacpro (F'lacI^{Q}, lacZ::Tn5, pro^{+}) (13). AT46 is AT44 (pAT45). Plasmid pAT45 is described below. Mu cts62 was used as the donor of Mu transposition functions. M13mp8 gyrB and M13mp19 gyrB contain a 3.7 Kbp XmaI-HpaI fragment covering most of the coding region of the gyrB gene cloned into M13mp8 (6) and M13mp19 (14) phage vectors, respectively. M13mp18 gyrB carries a 3.7 Kbp EcoRI-HpaI fragment (60 bp longer than the XmaI-HpaI fragment) cloned into the M13mp18 phage vector (14).

The mini-Mu plasmid pAT38 shown in Fig. 1 was constructed from three pieces. The vector part was modified from pMM306 (15) by replacing a 19 base pair AhaIII fragment (position 3232-3251 of the pBR322 sequence) with an XhoI linker (New England Biolabs). Mu end fragments were obtained by cutting the DNA of another mini-Mu plasmid, pMK108 (16, 17) with RsaI in the presence of purified Mu A protein, which protects an RsaI site at position 19 of the Mu L end (18). The 397 base pair RsaI fragment containing the L end was purified

and ligated to an XhoI linker. An RsaI fragment of about 290 base pairs containing the R end was purified from the same digest and ligated to an EcoRl linker (Collaborative Research). These Mu-end fragments were digested with XhoI or EcoRI and inserted at the appropriate restriction site on the vector DNA. Plasmid pAT45 was constructed from pAT38 and pMK208 (19) by substituting a small EcoRl - BamHl fragment of pAT38 containing the attB sequence of lambda with the EcoRl - BamHl fragment of pMK208 that contains lambda pL and the A and B genes of Mu. The structure of this plasmid is shown in Fig. 1. Strain AT46, which carries this plasmid, is very unstable presumably due to the substantial frequency of transposition of the mini-Mu caused by basal level expression of the Mu A and B genes. Attempts to stabilize the strain are in progress.

Enzymes and Oligonucleotides

DNA polymerase I (Klenow fragment) was obtained from IBI or Boehringer-Mannheim. Restriction endonucleases were obtained from New England Biolabs. T4 DNA ligase was obtained from Boehringer-Mannheim. T4 polynucleotide kinase was obtained from P-L Biochemicals. Mu A protein was purified as described (19). Deoxynucleoside triphosphates and dideoxynucleoside triphosphates were obtained from New England Biolabs. α-^{35}S-labelled deoxyadenosine α-thiotriphosphate (~ 1000 Ci/mmol) was obtained from New England Nuclear.

Several oligonucleotide primer sequences were synthesized by phosphoramidite chemistry in an Applied Biosystems 380 A automatic oligonucleotide synthesizer, and purified by using a reverse phase column (Waters μ bond pack C18 or Hamilton PRP-1) and ethanol precipitation. (We are indebted to Dr. G. Zon for his help in the synthesis of the oligonucleotides.) The following primer sequences were used (positions are numbered from the ends of Mu DNA):

L-end primer: TTTTCGTACTTCAAG (position 27 to 13).

R-end primer: TTTTCGCATTTATCGTG (position 49 to 33).

R-end primer: TTTTTCGTGCGCCGCTT (position 19 to 3).

R-end primer: GCATTTATCGTGAAACG (position 44 to 28).

Transposition of Mini-Mu Into M13 Clone DNA

The method used for preparing M13 clones with insertions of the mini-Mu transposon into the gyrB gene is shown schematically in Figure 2. The mini-Mu carrying strain AT40 or JM105 (pAT38) was grown to a cell density of 2 x 10^8/ml in L broth containing 2.5 mM $MgSO_4$ and 1 mM $CaCl_2$, at 37°C. A half-ml of the culture was mixed with Mu cts62 phage at a multiplicity of infection (moi) of 4 and incubated at 42°C for 15 min. M13mp8 gyrB, M13mp18 gyrB, or M13mp19 gyrB was added at a moi of 4 and the culture was aerated at 37°C for 2 hrs or

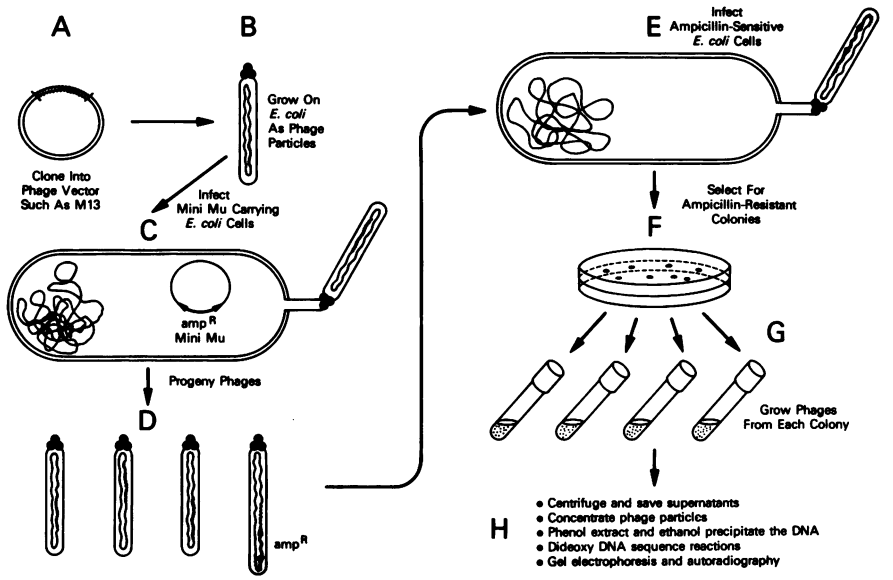


Fig. 2. Basic scheme for the use of mini-Mu transposition in DNA sequencing. See text for explanation.

until cell lysis occurred. A few drops of chloroform were added, the lysate was centrifuged at about 10,000 x g for 5 min, and the supernatant containing the phage was collected.

Two microliters of the phage lysate were mixed with 1 ml of a log-phase culture (2 x 10^8/ml) of AT32 (or AT33, AT42, or JM105 Mu^R) in L broth and left standing at room temperature for 30 min. One-tenth ml aliquots of the infected culture were plated onto L-agar plates containing 30 μg/ml of ampicillin for AT32 (or JM105 Mu^R), or 30 μg/ml each of ampicillin and chloramphenicol for AT33 or AT42, and the plates were incubated at 31°C overnight.

One-and-a-half ml aliquots of a 1:100 dilution of an overnight culture of AT32 (or JM105) in L-broth were distributed into test tubes. Each tube was also inoculated with a single colony of the amp^R (or amp^R cm^R for AT33) cells. The cultures were aerated vigorously for 5 hrs at 37°C. Cultures were centrifuged at 10,000 x g for 5 min and the supernatants containing M13 phages with the cloned fragment and mini-Mu inserts were retained. Only a limited number of mini-Mu insertions were found within the vector part of the M13 DNA, presumably due to the selection against inactivation of M13 functions. Mini-Mu insertions into the vector DNA were found in the small area between gene IV and the replication origin of M13 and in the lac gene fragment.

In some experiments, induction of the Mu genes carried on a plasmid was used to supply transposition functions, instead of infection with phage Mu. For this purpose, AT46 was grown to a cell density of about 2 x 10^8/ml in L broth containing 1 mM $MgSO_4$, at 31°C. The temperature was shifted to 42°C for 3 to 15 min and the culture was chilled in ice. M13mp18 gyrB or M13mp19 gyrB was added to a moi of 4 and the culture was aerated at 31°C for 2 hrs. The supernatant containing the phage was prepared and used as described above.

Preparation of the Viral DNA of M13 Clone with Mini-Mu Insert

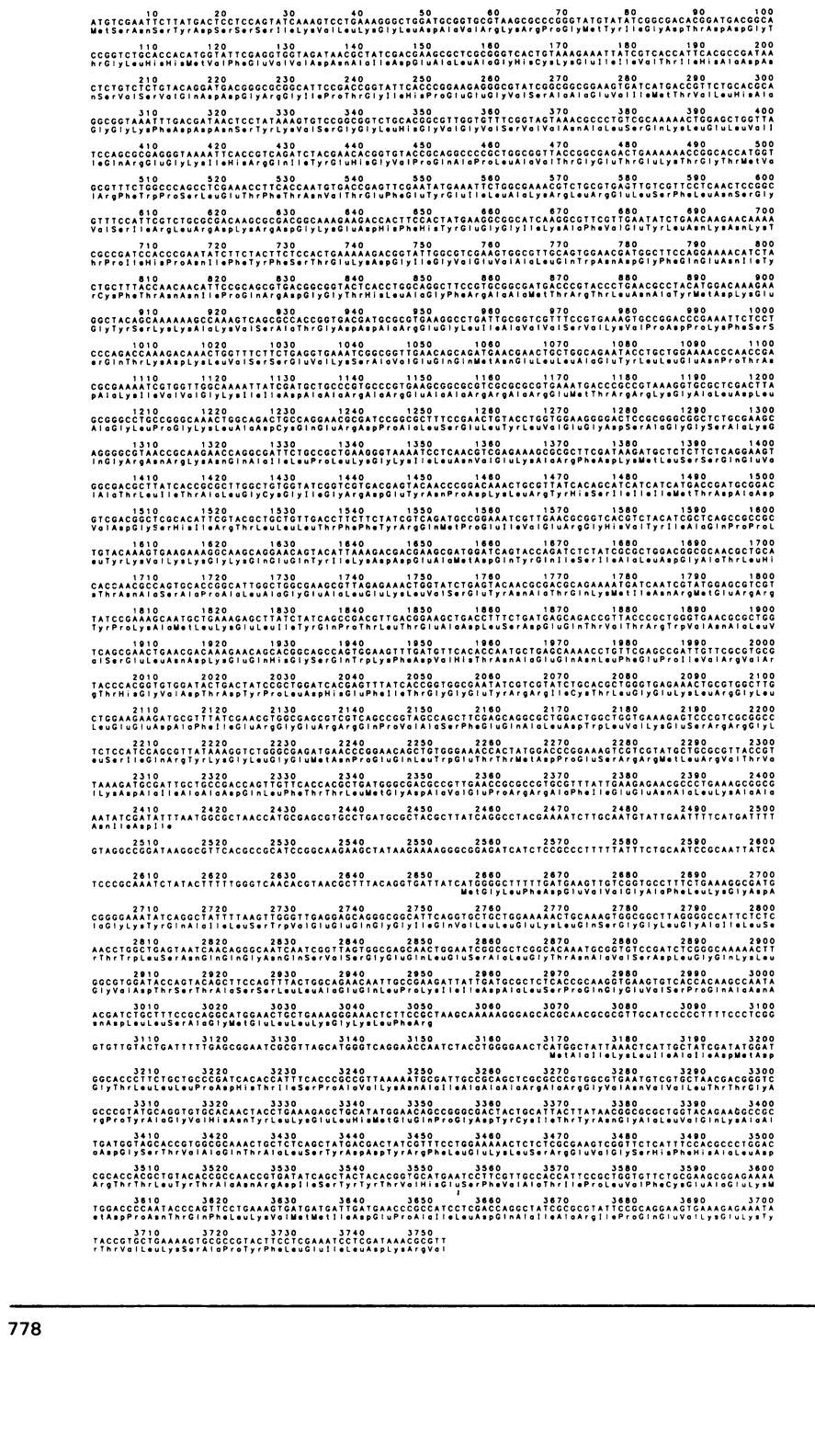
M13 phages with the cloned fragment and a mini-Mu insert were precipitated with polyethyleneglycol (PEG), extracted with phenol, and the DNA was precipitated with ethanol as described by Sanger et al. (4).

Determination of the Location of Mini-Mu Insertion Sites

After most of the gyrB sequence had been determined, it was advantageous to pre-screen the mini-Mu inserts for the desired insertion sites and use only those with a mini-Mu insertion near those parts of the sequence which needed confirmation or extension. The insertion sites can be determined by digesting the replicative form DNA of the clone with an appropriate pair of restriction enzymes and sizing the fragments. However, to avoid the preparation of RF DNA for each clone, we devised another method. The viral DNA of each clone was annealed with viral DNA of another clone containing the opposite strand of gyrB DNA, either with a mini-Mu insertion at a known location or without an insertion. The annealed DNA was digested with mung bean nuclease (10 to 50 units of the enzyme per 10 µl reaction in 0.2 M NaCl, 50 mM Na acetate, pH 4.8, 1 mM $ZnSO_4$, 0.5% glycerol at 37°C for 30 min) and the size of the double-stranded DNA fragments was determined by agarose gel electrophoresis. Double-stranded DNA fragments corresponding to the gyrB sequence cut at each mini-Mu insertion site were observed.

Sequencing Reaction

The primer extension method of Sanger et al. (8), involving a set of base specific chain terminators, was used essentially as described. Both an L-end primer and an R-end primer (described above) were added together (3-4 ng each per set of four 6 µl reactions). In some cases, the orientation of the mini-Mu insert was determined prior to the sequencing reaction; in these cases, only one primer complementary to the proper end was added. ^{35}S-labelled deoxyadenosine α-thiotriphosphate (5 µCi per reaction) was used as the radioactive precursor. The reaction products were separated by denaturing acrylamide gel electrophoresis (20). The random segments of the sequence were



assembled by making use of a computer program developed by Staden (3).

Determination of N-terminal Amino Acid Sequence

Edman degradation was carried out on an Applied Biosystems 470 amino-acid sequencer. PTH derivatives were analyzed on a Hewlett-Packard 1090A liquid chromatograph, using a DuPont Zorbax ODS column.

RESULTS AND DISCUSSION

In determining the sequence of the EcoRl - HpaI fragment, which covers a stretch of 3748 base pairs, we used about 80 independent insertions of the mini-Mu into this region. From each insert, a sequence of 200-300 bases could be determined; the redundancy needed for the assembly of the random array of fragments into a continuous sequence was thus about 5-fold, including confirmation of the entire sequence from the opposite strand. This level of redundancy allows for reasonably efficient sequence determination with a high degree of certainty. The DNA sequence is shown, together with the corresponding amino acid sequences of the open reading frames, in Figure 3. The recognition sites of six-base sequence specific restriction enzymes are summarized in Table 1.

Open Reading Frames

The sequence of the N-terminal part of the gyrB gene, up to position 319, was previously determined, and the start point was identified by comparing the DNA sequence with the N-terminal amino acid sequence of the Gyr B protein (21). (Nucleotide positions are numbered from the first A of the gyrB initiation codon as position 1.) The N-terminal methionine was shown to be missing from the protein. The coding sequence begins five bases before the EcoRl site at the left end of the region sequenced in the present work. A few mistakes were found in the previously published sequence at positions 151, 152, 167 and 168.

The sequence determined here continues the open reading frame to a total length of 804 amino acids and corresponds to a protein of molecular weight 89,835 (corrected for the removal of the N-terminal methionine), in good agreement with earlier estimates of 90 - 95,000 (22, 23, 24). The sequence of the gyrB gene has just been determined independently by Yamagishi et al. (25). The two sequences are identical except that at position 1584 in our numbering, a C in our sequence is replaced by a T in theirs. The differ-

Fig. 3. DNA sequence of E. coli gyrB coding region and its downstream region. Open reading frames are indicated by their corresponding amino acid sequences.

Table 1. Positions of six base-pair restriction enzyme recognition sites.

AatII	1498	MluI	3746
AsuII	544	NcoI	494
BclI	278, 1778	NdeI	3339
BglI	454, 1205	NruI	3467
BglII	428, 1667	PvuII	2245
BssHII	1160, 1366, 3379	SacII	1280
BstEII	465	SalI	1501
BstXI	2333, 2923, 3568	StuI	951, 2458
ClaI	1126, 2404, 3189	XmaI	66
EcoRI	6	XmnI	714, 3038
EcoRV	3528		

The following six-base or eight-base restriction enzyme recognition sites do not appear within the sequence shown in Fig. 3: AhaIII, ApaI, BalI, BamHI, HindIII, HpaI, KpnI, MstI, MstII, NaeI, NarI, NotI, PaeR7I, PstI, PvuI, SacI, ScaI, SfiI, SphI, Tth111I, XbaI, XhoI, XmaIII.

ence is in the third position of a tyrosine codon; the amino acid sequences are identical.

The sequence was scanned for other open reading frames (ORF's) corresponding to possible proteins longer than 100 amino acid residues. Downstream of gyrB and reading in the same direction, there is an ORF of 132 amino acid residues (nucleotides 2655-3050), and an ORF of at least 195 residues (nucleotide 3168 to the end of the sequence at 3752), both starting with an ATG codon and preceded by a reasonable ribosome binding site. In the opposite direction, there is only the end of an ORF, reading into the sequenced region from beyond position 3752 up to 3291, a length of 154 amino acids. It is not clear whether any of these ORF's corresponds to a known protein. The gene for the protein component of ribonuclease P (rnpA), formerly thought to lie near the downstream side of gyrB, (reviewed by Bachmann and Low (26)), has recently been more precisely mapped to a position some distance away, on the opposite side of dnaA (27). No other gene is known to be very close to the downstream side of gyrB.

Transcription of gyrB

A transcription start point at position -31 was previously identified

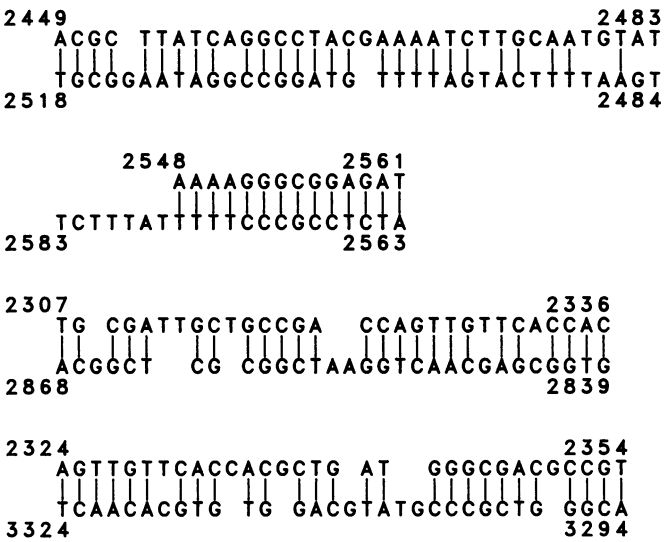


Fig. 4. Sequences with dyad symmetry at the end of gyrB gene. The sequences are shown in their potential fold-back structures with bars indicating the complementary bases.

(21). When the promoter region corresponding to this initiation site was cloned into a plasmid containing the galactokinase gene, it was shown to confer transcription regulated by DNA supercoiling in the same way as the transcription of gyrB normally is. This is most probably the promoter used for transcription of gyrB in vivo. A search for other potential promoter sequences was carried out by making use of the program developed by Mulligan et al. (28). Four sequences were found with promoter scores greater than 50% and a spacing between -35 and -10 regions of 16 to 19 nucleotides; the midpoints of the -10 regions are located at positions 308 and 2497 for the forward orientation and positions -21 and 167 for the reverse orientation. The physiological significance of these potential promoters is not clear at present.

A plausible termination sequence can also be identified. In analyzing the sequence for self-complementary stretches, we noted a pair of strongly favored stem-loop structures just beyond the 3' end of gyrB. One region, spanning positions 2449 to 2518, contained 27 base pair matches in a stretch of 34 nucleotides (Fig. 4). Shortly after this (positions 2548 to 2576), there is a region with 14 consecutive base pair matches; at the 3' end of this stem, there is the sequence TTTTTATTTCT, which is similar to that found in

Table 2. Amino acid composition of Gyr B protein.*

Ala	67	Leu	70
Arg	51	Lys	52
Asn	31	Met	19
Asp	53	Phe	24
Cys	5	Pro	28
Gln	32	Ser	42
Glu	68	Thr	44
Gly	61	Trp	6
His	21	Tyr	26
Ilu	48	Val	55

*The sequence-derived composition has been corrected for removal of the N-terminal methionine.

many rho-independent termination sites. This stem-loop is very likely to mark the termination of gyrB mRNA. These sequences are themselves embedded in another pair of possible stem-loop structures. In one, nucleotides 2307-2336 are paired with nucleotides 2839-2868; in the other, nucleotides 2324-2354 are paired with nucleotides 3294-3324 (Fig. 4). The significance of these pairing possibilities is obscure, but it is possible that, as in the phage λ sib situation (29), such stem-loops could be used as sites to initiate RNase III-promoted degradation of those mRNA molecules which fail to terminate at the proper site.

Amino Acid Composition and Codon Usage

The amino acid composition of the Gyr B protein, derived from the DNA sequence, is given in Table 2. It predicts an isoelectric point slightly on the acidic side of neutrality, in agreement with results from isoelectric focusing (our unpublished results).

Codon usage was analyzed according to the treatment of Ikemura and Ozeki (30). The frequency of use of optimal codons in Gyr B was 0.78, a moderately high value typical of proteins expressed at a level of about 10^3 copies per cell, as is the case for Gyr B (22, 31, 32).

Location of Enzymatically Active Gyr B Fragment in the Sequence

A protein of molecular weight about 50,000, which combines with the Gyr A

protein to give a DNA-relaxing activity, has been purified from E. coli extracts (33, 34). The protein was identified by partial proteolysis as a fragment of the Gyr B protein (33). We have now determined the N-terminal amino-acid sequence of this fragment; it is arg lys gly ala leu asp leu ala gly leu pro gly lys leu ala asp. Comparison with the sequence of the whole Gyr B protein shows that exactly this stretch of amino acids occurs beginning at amino acid position 394. A protein beginning at this position and extending to the C-terminus of Gyr B would have a molecular weight of 46,582, in good agreement with the estimate of 50,000 for the Gyr B fragment. This fragment is thus essentially the C-terminal half of Gyr B. It must contain sites for binding to the Gyr A protein, and carry whatever information is necessary for Gyr A, which is the DNA breaking-rejoining subunit of DNA gyrase (see ref. 1 for review), to become an active topoisomerase. On the other hand, neither the Gyr B fragment nor its complex with Gyr A and DNA has a measurable ATPase activity (33), whereas the whole Gyr B protein has a low level of ATPase activity which is greatly stimulated by Gyr A and DNA (35). The ATPase site of Gyr B must be (at least partly) contained in the N-terminal half of the protein. Now that the complete sequence of Gyr B is available, it should be possible to locate different functions of the enzyme more precisely.

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