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DNA gyrase binds to the family of prokaryotic repetitive extragenic palindromic sequences

(repetitive extragenic palindromic element/gel electrophoretic mobility-shift assay/oxolinic acid/site-specific DNA cleavage/nucleoid)

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ABSTRACT **A family of repetitive extragenic palindromic (REP) sequences is composed of hundreds of copies distributed throughout the chromosome. Their palindromic nature and conservation suggested that they are specifically recognized by a protein(s). We have identified DNA gyrase [DNA topoisomerase (ATP-hydrolysing), EC 5.99.1.3] as one of the REP-binding proteins. Gyrase has at least a 10-fold higher affinity for DNA containing REP sequences than for DNA not containing REP sequences. Binding effectiveness correlates directly with the number of REP sequences in the DNA. DNase I footprinting shows that gyrase protects 205 base pairs on a REP-containing DNA fragment enclosing the REP sequences. In agreement with the above results, a comparison of the REP consensus sequence with the sequence of previously identified pBR322 "strong" gyrase cleavage sites reveals a high degree of homology. Because REP sequences are numerous and found throughout the genome, we suggest they have physiological functions mediated through their interaction with gyrase, such as being sites of action for the maintenance of DNA supercoiling. In addition, we speculate that these interactions may be of a structural nature, such as involvement in the higher-order structure of the bacterial chromosome.**

A family of repetitive extragenic palindromic (REP) sequences has been discovered in the chromosome of *Escherichia coli* and *Salmonella typhimurium* (1, 2). The REP family is composed of hundreds of copies per genome, which are distributed throughout the chromosome and are always located outside structural genes. It has been estimated that these sequences comprise ≈0.5% of the chromosomal DNA. A consensus sequence has been obtained by analyzing 35 REP sequences. REP sequences often appear in clusters of two, three, or four—each cluster being referred to as a REP element. REP elements, which may constitute the biological unit of REP function, are estimated to be present in about one to two hundred locations per chromosome. Given this abundance and considering the economy of DNA use in the prokaryotic chromosome, REP sequences probably perform an important function. They have been postulated to be involved in regulating intraoperonic gene expression and, indeed, they have been found to be involved in protection of mRNA from degradation and in modulation of translational initiation (3, 4). However, both effects can be explained as a simple consequence of the palindromic secondary structures forming in mRNA, and extensive sequence homology is usually not found among RNA secondary structures involved in a generalized function (e.g., ρ-independent transcription terminators and RNase protection structures), whereas the family of REP sequences maintains remarkable homology. We therefore searched for additional REP activities that might reveal their function. In particular, the palindromic nature and conservation of REP sequences suggested that they are specifically recognized by a protein(s). A search for REP-binding proteins in cell extracts yielded several possible candidates, and we show here that one of these REP-binding proteins is the enzyme DNA gyrase [DNA topoisomerase (ATP-hydrolysing), EC 5.99.1.3]. [A REP-binding activity in chromoid-associated protein extract has been described (5). Its relationship to our results is unclear.]

Bacterial DNA gyrase is an essential protein, the activities of which *in vitro* include negatively supercoiling, catenating, and decatenating of circular DNA (6). Despite several attempts to determine the site on DNA recognized by gyrase, no clear-cut recognition sequence has been identified (7). Gyrase is thought to have multiple functions, such as involvement in DNA replication and repair, recombination, transposition, control of transcription, chromosome segregation (6, 8), and the maintenance of bacterial nucleoid structure (8). Most of these functions may be directly related to the level of DNA supercoiling, which is thought to be regulated by the opposing actions of topoisomerase I and of gyrase. The correlation between gyrase activity and these functions has been studied *in vivo* using gyrase mutants (9) or gyrase inhibitors (10). However, it is difficult to determine immediate cause and effect relationships, because alterations in gyrase activity are, expectedly, very pleiotropic. Thus, it has not been possible to identify the chromosomal site(s) of gyrase action. Our finding that DNA gyrase binds to REP sequences suggests that they might be important sites of gyrase action *in vivo*.

MATERIALS AND METHODS

Proteins. Gyrase (2×10^5 units per mg) was purified from strains N4186 and MK47, reconstituted (11) and used in up to 4-fold molar excess over DNA.

DNA. pFA20 (12) has a REP-containing DNA fragment [337 base pairs (bp); *Hin*dIII–*Alu* I] from the intergenic region *his*(*J–Q*) of the histidine transport operon of *S. typhimurium* between the *Eco*RI and *Hin*dIII sites of pKK177-3. pFA21 (12) has a DNA fragment from the histidine transport operon that does not contain a REP sequence (358 bp; *Hin*dIII–*Alu* I) between the same sites of pKK177-3. pFA55 (12) has a synthetic consensus REP fragment (44 bp) in the *Sal* I site of pKK177-3. pFA99 has a REP-containing DNA fragment (304 bp; *Ssp* I–*Cla* I) from pGS64 (13) in the *Sma* I site of pUC19. Four probes were used for the electrophoretic mobility-shift assay. (*i*) A synthetic 44-bp consensus REP sequence (1, 12). (*ii*) Two natural REP-containing DNA probes (218 and 344 bp, respectively) were prepared from pFA20 by digestion with either *Rsa* I or *Eco*RI and *Hin*dIII. (*iii*) Another natural REP-containing DNA probe (304 bp) was prepared by digestion of pGS64 with *Cla* I and *Ssp* I. Probes for gyrase cleavage and DNase I footprinting experiments were 344-bp fragments

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Abbreviation: REP, repetitive extragenic palindromic.

prepared from pFA20 3′ end labeled at either *Eco*RI or *Hin*dIII. All probes were end labeled by standard methods (14). Two DNA fragments containing no REP sequences and no known gyrase-cleavage sites were prepared as nonspecific competitor DNA: (*i*) a fragment of 183 bp (between bp 611 and 793 of *hisG*; ref. 15) was generated by the polymerase chain reaction and (*ii*) a fragment of 218 bp was prepared by digesting pGS64 with *Ssp* I and *Cla* I. A fragment of 182 bp containing a consensus REP sequence was prepared by digesting pFA55 with *Eco*RI and *Ava* II. A fragment of 359 bp containing four REP sequences was prepared by digesting pFA99 with *Eco*RI and *Hin*dIII. Poly(dI–dC)·poly(dI–dC), of average size 2 kilobase pairs (kbp), was purchased from Pharmacia.

Assays. The gel electrophoretic mobility-shift assay (16–18), gyrase-mediated DNA cleavage (19), and DNase I footprinting analysis (20, 21) were performed as described.

RESULTS

DNA Gyrase Binds to REP-DNA. Incubation of a 218-bp DNA fragment containing two natural REP sequences (i.e., a REP element) with cell extract results in the formation of a protein–DNA complex in mobility-shift assays. Incubation of the same fragment with purified gyrase yields a complex with the same mobility (data not shown). This and immunological data (unpublished data) suggest that the complex formed by cell extract is due to gyrase. The affinity of purified gyrase for REP sequences was estimated by a series of competition studies. The amount of the complex was reduced by 50% at 1000-fold excess (wt/wt) of poly(dI-dC)·poly(dI-dC) concentrations. Competition studies with plasmid DNA digested with *Hae*III showed that >10-fold excess of DNA not containing REP (pFA21 or pBR322) over DNA containing REP sequences (pFA20) was needed to eliminate the complex (data not shown). Assuming that a molecule of pFA20 (3203 bp) contains two high-affinity sites, whereas a molecule of pFA21 (3231 bp) or pBR322 (4363 bp) contains 3000 or 4000 nonspecific sites, the apparent affinity ratio for specific vs. nonspecific sites is greater than $(3000/2) \times 10 = 1.5 \times 10^4$.

Because REP sequences often appear in clusters of two, three, or four, we investigated whether the affinity of binding to gyrase depends on the number of REP sequences. We used two probes, containing either two or four REP sequences, 218 bp and 304 bp, respectively. Fig. 1*a* shows that at very low gyrase concentration the 304-bp probe forms two complexes (A and B; lane 1), whereas the 218-bp probe forms none (lane 14). This indicates a higher affinity of gyrase for four than for two REP sequences. Increasing concentrations of gyrase result in the formation of only the A complex from the 304-bp probe (lanes 2–4) or of a complex with the 218-bp probe (C; lanes 11–13). Adjusting the reaction conditions to contain both DNA probes and limiting gyrase (20 ng) allows formation of all three complexes (lane 10). This suggests that the stability of complex A is lower than that of complex B and that complexes B and/or C are formed at the expense of complex A (compare lanes 3 with 10). Addition of poly(dI-dC)·poly(dI-dC) eliminates complex C preferentially, as compared with complex B (lanes 5–9), thus confirming that the affinity of gyrase for four REP sequences is higher than for two REP sequences.

Fig. 1*b* shows that plasmid pFA20 (two REP sequences) competes with the 218-bp fragment about 10 and 5 times more effectively than pFA21 or pBR322, which have no REP sequences (e.g., compare lanes 3 and 14, and lanes 4 and 8, respectively). Supercoiled pBR322 is a poor competitor, as compared with *Hae*III-digested pBR322 (e.g., compare lanes 7 and 11). In addition, since the pBR322-derived vector (pKK177-3) used in the construction of both pFA20 and pFA21 contains several strong gyrase-cleavage sites [such as sites at 2384, 2472, and 3689 (ref. 7)] and, therefore, presumably contains at least three high-affinity binding sites, these competition results suggest that the gyrase has at least a 15-fold ($3/2 \times 10$) higher affinity for REP sequences than for those cleavage sites.

Competition by increasing amounts of each of four DNA fragments carrying either no, one, two, or four REP sequences shows that the higher the number of REP sequences, the more effective is the competition (Fig. 1*c*). A fragment that does not contain REP sequences does not compete at the highest level used (lane 13). To eliminate the possibility that the size of the DNA fragment has an effect on its affinity for gyrase, we have used a variety of labeled fragments as probes (304-bp fragment with four REP sequences; the 218-bp and the 344-bp fragments with two REP sequences) and several fragments of a variety of sizes (from 182 bp to 359 bp) as competitors. Competition effectiveness in all cases correlated with the number of REP sequences present and not with

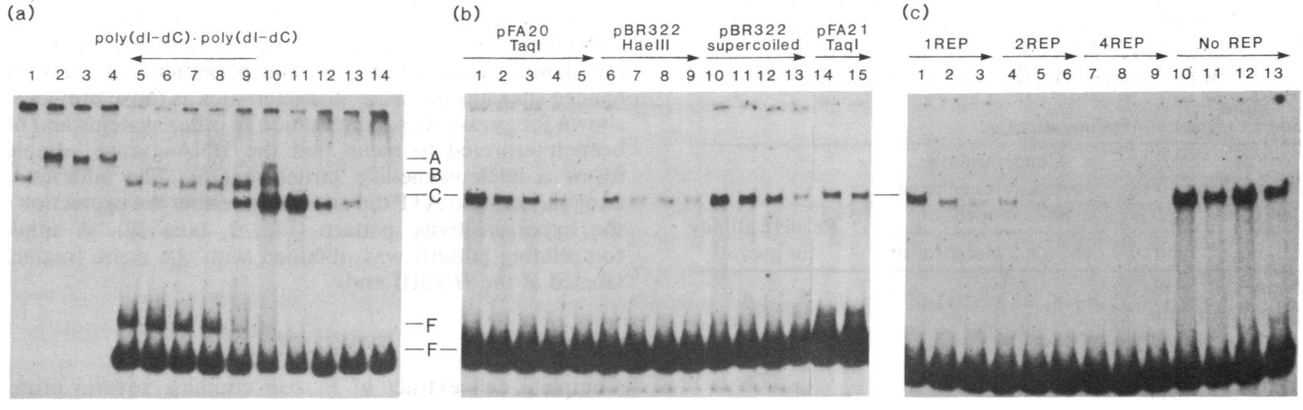

FIG. 1. Competition analysis of gyrase binding. Reaction mixtures contained 1.5 ng of labeled DNA fragments, varying amounts of competitor DNA (as shown above each set of competitions), and 20 ng of gyrase, except where indicated. A, B, and C, shifted bands that are gyrase–REP complexes; F, free probes. (*a*) Reactions contained labeled fragments: 304 bp (lanes 1–10) and 218 bp (lanes 5–14), and varying amounts of poly(dI-dC)·poly(dI-dC): 100 ng (lane 5), 50 ng (lane 6), 25 ng (lane 7), 10 ng (lane 8), and 5 ng (lane 9). Lanes: 1 and 14, 5 ng of gyrase; 2 and 13, 10 ng of gyrase; 4 and 11, 40 ng of gyrase. Complex A is probably due to additional molecules of gyrase binding to complex B. (*b*) Reactions contained labeled fragment (218 bp) and the following amounts of competitor DNA (digested as indicated): 25 ng (lane 1), 50 ng (lane 2), 75 ng (lane 3), 100 ng (lanes 4, 6, and 10), 250 ng (lanes 5, 7, and 11), 500 ng (lanes 8 and 12), 750 ng (lane 14), 1000 ng (lanes 9, 13, and 15). (*c*) Reactions contained labeled fragment (218 bp) and the following amounts of competitor DNA fragments carrying one (182 bp), two (344 bp), four (359 bp), or no (218 bp) REP sequences: 25 ng (lanes 1, 4, 7, and 10), 50 ng (lanes 2, 5, 8, and 11), 100 ng (lanes 3, 6, 9, and 12), and 250 ng (lane 13).

the size of the fragments. In particular, experiments using a 218-bp fragment containing two REP sequences as the probe and either the same nonlabeled fragment as the specific competitor or a DNA fragment of 218 bp containing no REP sequence as the nonspecific competitor showed that about 15-fold excess of the nonspecific competitor DNA over the specific competitor DNA was needed to completely antagonize complex formation. Table 1 summarizes these results: clearly, gyrase binds progressively better to DNA containing one, two, and four REP sequences, in that order, and much better than to DNA containing no REP sequence, independent of the size of the fragment.

Interestingly, gyrase A subunit alone also binds specifically to REP-containing DNA fragments (both the 218- and 44-bp fragments); the interaction was shown to be REP-specific by competition experiments with fragments carrying either none, one REP sequence, or four REP sequences, or the gyrase cleavage site on pBR322 at 990 bp (7). The complex, which migrated faster than complex with the complete gyrase, was confirmed to contain only subunit A and no subunit B by immunoblotting with antibodies raised against either the A or the B subunit (data not shown). Consistent with our results is the finding that DNA binding by subunit A alone was demonstrated by electron microscopic studies (22, 23).

Site-Specific DNA Cleavage by Gyrase. Because gyrase is thought to cleave DNA at its interaction site when the complex is exposed to the gyrase inhibitor, oxolinic acid, and a protein denaturant (6, 19), we used this method to determine its site of cleavage on a 344-bp (*Eco*RI–*Hin*dIII) fragment containing two REP sequences. Fig. 2 shows that cleavage occurred at eight sites, with one considerably more prominent than the others, yielding a 190-bp fragment. At the shortest incubation times and/or with the smallest amounts of gyrase, the 190-bp fragment is at least 5-fold more abundant than the next most abundant fragment (140 bp). To analyze the level of cleavage specificity further, cleavage at these sites was examined in the presence of poly(dI-dC)·poly(dI-dC): at a weight ratio of up to 15,000-fold, this competitor did not prevent completely the production of the 190-bp fragment (lane 4 of Fig. 2). These results indicate the existence of a strongly preferred site, which has been identified as being located immediately outside the REP element (see footprinting, Fig. 3). The other sites are located mostly within the REP element. Several secondary cleavage sites have also been observed in other systems (20, 24, 25). The location of the cleavage sites was independent of the size of the fragment used (the same cleavage sites were obtained from a 218-bp smaller DNA fragment containing the same

Table 1. Relative binding affinities

Competitor DNA		Concentration necessary to achieve 50% competition		Relative affinity for gyrase†
REP, no.	bp	nM	Molar ratio*	
None	183 218	>348	>31.6	1.0
1	182	≈58	5.3	6.0
2	218 344	≈28	2.5	12.6
4	304 359	≈11	1.0	31.6

*Molar ratios of each fragment necessary to achieve 50% competition, normalized to the molarity of the fragment containing four REP sequences.

†DNA probe used is the 218-bp fragment with two REP sequences. Relative binding affinity is normalized to the affinity of gyrase for a DNA fragment containing no REP sequences and no known gyrase-cleavage sites.

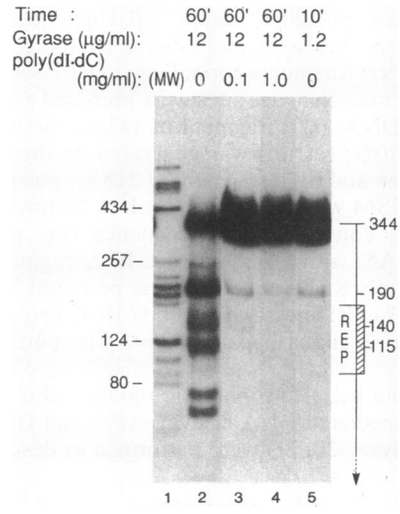

FIG. 2. Cleavage of REP–DNA by gyrase. Reaction mixtures contained DNA at 0.65 μg/ml (344 bp) labeled at the *Eco*RI end, 0.2 mM oxolinic acid, and 1.6 mM ATP in 10 μl. Gyrase and poly(dI-dC)·poly(dI-dC) were present as indicated. Lane 1, *Hae*III-digested pBR322.

REP element; data not shown) or the presence of ATP or variations in oxolinic acid concentration. The same cleavage sites were also detected in a plasmid containing the same 344-bp fragment, either in the supercoiled state or digested with a restriction enzyme to yield four fragments (data not shown). The existence of a strongly preferred site indicates that gyrase has a defined specificity in its interaction with REP-containing DNA. *In vivo* evidence that this interaction is specific was obtained by demonstrating that *in vivo* cleavage of a REP-containing plasmid occurs preferentially at the REP element (Y. Chen, Y.Y., and G.F.-L.A., unpublished data).

Footprinting of the REP-Gyrase Complex. DNase I footprinting of the 344-bp (*Eco*RI–*Hin*dIII) fragment was used to identify the sequences specifically bound by gyrase (Fig. 3). Fig. 4 summarizes the footprinting and cleavage results and correlates them with the DNA sequence. Gyrase protected a region of ≈200 bp, encompassing the REP sequences (located in the center) and the known cleavage sites. Clearly visible throughout the footprint are DNase I-hypersensitive sites, which are spaced ≈10 bp apart. Of these, the two sites with the highest sensitivity are located close to the boundaries of the 200-bp protected region. The periodicity of these enhanced sites has the same characteristics as those previously shown for gyrase–DNA interaction in other systems and has been interpreted to mean that the DNA–gyrase complex forms a nucleosome-like structure (26). The addition of oxolinic acid and ATP did not change either the protection or the hypersensitivity pattern (Fig. 3, lane 12). A similar footprinting pattern was obtained with the same fragment labeled at the *Hin*dIII end.

DISCUSSION

Complete cell extract of *E. coli* contains several protein factors capable of binding to DNA fragments containing either a consensus REP sequence or natural REP sequences (41). Here we show that DNA gyrase, one of the known *E. coli* DNA-binding proteins, binds specifically to REP sequence. Furthermore, one of the complexes detected in cell extract is formed by interaction with gyrase (unpublished data). Because there are ≈500–1000 molecules of each gyrase subunit per cell (M. Gellert, personal communication), gyrase must have a high affinity for REP as compared with other

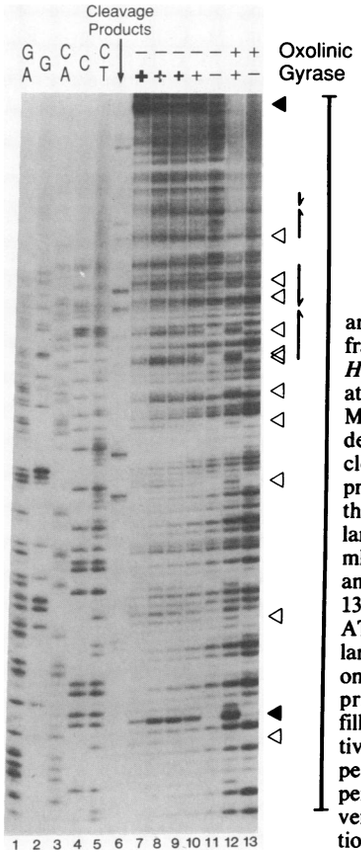

FIG. 3. DNase I footprint analysis. The REP-containing fragment was the 344-bp *Eco*RI–*Hin*dIII fragment 3′ end labeled at the *Eco*RI site. Lanes 1–5, Maxam–Gilbert sequence ladder; lane 6, gyrase-mediated cleavage products. The footprinting reaction mixes contain the following amounts of gyrase: lane 7, 7.5 μg/ml; lane 8, 3.8 μg/ml; lane 9, 1.9 μg/ml; lanes 10 and 12, 0.9 μg/ml; lanes 11 and 13, 0. Oxolinic acid (0.2 mM) and ATP (1.6 mM) are present in lanes 12 and 13. The vertical line on the right encompasses the protected region. Open and filled triangles indicate, respectively, the location of sites hypersensitive and unusually hypersensitive to DNase I. The vertical arrows indicate the location of the two REP sequences.

proteins in the cell extract in order to bind them. Indeed, we have shown that REP sequences are specifically recognized by gyrase: REP sites are bound with an affinity at least 1.5×10^4 times that of non-REP sites. The presence of numerous REP elements per cell increases the probability of their interaction with gyrase (27). (If we assume that in the cell 80% of all bound gyrase molecules is bound specifically and that 60% of REP locations is occupied by gyrase, then the preference of gyrase for REP sites versus nonspecific sites would be $\approx 10^5$-fold according to the equations discussed in ref. 27. This is not inconsistent with our value of 1.5×10^4.) The footprinting results allow a preliminary estimate of the strength of binding: about 50% protection is afforded at a gyrase concentration of $\approx 4 \times 10^{-9}$ M. The ability of gyrase to bind to a very small REP-containing fragment (44 bp) may also be an indication of high affinity for REP sequence (unpublished data), because no complex formation has been previously detected by a filter-binding assay with fragments <77 bp or by mobility-shift assay with fragments <55 bp (18).

Gyrase interacts with a large portion of DNA (205 bp), which includes an entire REP element (composed of two REP sequences) and neighboring areas. Specificity of interaction is also indicated by the fact that the REP element is centrally located within the protected region, despite the fact that it is asymmetrically located on the 344-bp DNA. The size of the protected area is larger than previously reported for other gyrase–DNA systems, which showed that gyrase protects 102–155 bp of DNA from DNase I (20, 24, 25, 28). The simplest interpretation of the larger protected region is that the complex formed with the 344-bp fragment contains two gyrase molecules because it contains two REP sequences. In fact, this fragment can form two complexes with gyrase, as determined by mobility-shift assay: the slow-moving complex might be generated by the binding of additional gyrase molecule(s) to the fast-moving complex (data not shown).

The major oxolinic acid-induced cleavage in our system occurs at a considerable distance (≈80 bp) from the center of the protected region, which is located between the two REP sequences. It has been suggested previously (28) that cleavage occurs within the central region of the protected area, where the gyrase topoisomerization site is presumed to be located. If the protection pattern reflects the binding of two gyrase molecules, the major cleavage site would be only slightly asymmetric relative to one of the two bound gyrase molecules. Cleavage has been shown to occur somewhat asymmetrically in several other systems (20, 24, 25). In addition, it has been shown that interactions involving flanking DNA are required to ensure efficient DNA scission at the cleavage site (29). In this respect, it is interesting that a good gyrase-binding site need not necessarily be cleaved (28). Analysis of oxolinic acid-induced cleavage sites in pBR322 *in vivo* (7) has yielded a set of rules for the cleavage site. However, the derived consensus sequence is somewhat vague, because 15 of its 20 positions are degenerate, presumably because inclusion in the analysis of the many "weaker" cleavage sites in pBR322, which may reflect poor affinity, distracts attention from the real consensus sequence. This sequence has little similarity to the REP consensus sequence. Therefore, we aligned the individual sites identified in ref. 7 with the REP consensus sequence (1, 12), which revealed that it has significant homology with the "strongest" cleavage sites. For example, pBR322 site at 990 is 70% (16/23) homologous to the REP consensus (bottom line of display):

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CTGgAT--GGCcTtCcCCaTTAT
CTG-ATgcGGCgTgCgCC-TTAT
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Sites at 1460 and 2472 can also be aligned with the REP consensus, giving 73% (16/22) and 63% (22/35) identity, respectively. Interestingly, gyrase was shown to interact with the plasmid pSC101 *par* locus (30). Inspection of the *par* locus sequence of pSC101 reveals regions of homology with various areas of a REP element, both within the REP sequences proper and in neighboring sequences. The significance of such homologies is unclear at present. In our hands, intro-

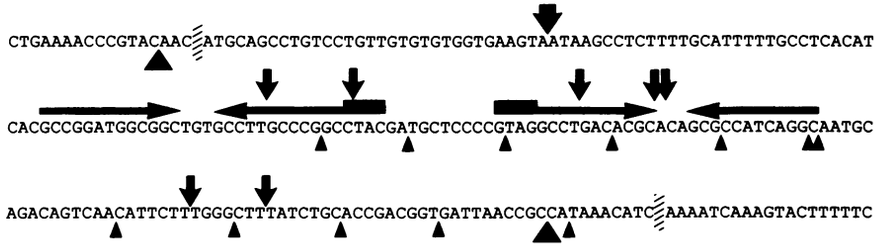

FIG. 4. Summary of interactions between the 344-bp REP-containing fragment and gyrase. Vertical arrows, sites of gyrase-mediated DNA cleavage; the bigger arrow denotes the major cleavage site. Triangles, locations of DNase I-sensitive sites; bigger triangles denote higher sensitivity. Hatched bars indicate the boundaries of the protected area. Horizontal arrows indicate the location of the REP sequences; the thicker portion denotes the "tail", which does not participate in the palindrome (1).

duction of a REP element into a plasmid deleted for a portion of its *par* locus resulted in a slight restoration of partition ability (12).

Because our data show that a specific interaction occurs at the REP sequence and because it is stronger than that at previously identified gyrase cleavage sites in pBR322, we hypothesize that REP sequences may be the physiological site(s) of gyrase action on the chromosome. Several important questions can be asked concerning the physiological meaning of the REP–gyrase interaction. In particular, we are interested in exploring its possible relationship to the architecture of the chromosome, which is organized in about 100 individually supercoiled domains (31) that must be anchored to each other and/or to the membrane by way of specific interactions at the base of the loops. Each chromosomal domain conceivably carries at least one specific gyrase-binding site (10). It is possible that REP sequences are the natural, preferred chromosomal binding sites for gyrase action in maintaining the appropriate level of negative supercoiling. The postulated number of gyrase-binding sites (corresponding to the number of domains) is in reasonable agreement with the estimated number of REP elements per chromosome (100–200). It is possible that the gyrase–REP interaction underlies a more complex, structural function, involving gyrase–gyrase (and probably other proteins) interactions linking one REP element to another and/or to the membrane. In this respect it is relevant to point out that the clustering of REP sequences into REP elements may indicate that they form complex structures analogous to those resulting, for example, from λ *int* function (32). Several pieces of evidence speak in favor of the above speculations: (*i*) it has been shown that gyrase mutants form defective nucleoids (9); (*ii*) oxolinic acid/NaDodSO$_4$ treatment *in vivo* fractures the chromosome, presumably at gyrase-binding sites, into at least 45 fragments, which might be the result of individual breaks occurring within each domain (10); (*iii*) REP elements are preferred sites of gyrase-mediated DNA cleavage *in vivo* (Y. Chen, Y.Y., and G.F.-L.A., unpublished data); (*iv*) gyrase is a component of the nucleoid (unpublished data); (*v*) gyrase can bind and bring together two separate DNA sites, as demonstrated by electron microscopic studies (22); (*vi*) the gyrase A sequence (33) contains three "leucine zippers"—i.e., leucine repeat motifs (residues 34–55, 410–424, and 447–489) recently identified in several DNA-binding proteins and hypothesized to be involved in the formation of DNA–protein scaffolds and in protein dimerization (34); and (*vii*) eukaryotic topoisomerase II, which is homologous to bacterial gyrase and may perform an analogous function, is a major structural component of the chromosomal scaffold in interphase nuclei and mitotic chromosomes (35, 36) and appears to be located at the base of the chromatin loops (37, 38). Thus, the elementary structure of the bacterial chromosome may be similar to that of eukaryotic chromosomes. Another possible function for a gyrase–REP interaction is that REP sequences are sites of chromosomal rearrangements resulting from gyrase action, because gyrase can effect illegitimate recombination (39). Indeed, we have shown that duplications can occur by recombination between distantly located REP sequences (E. Schneider and G.F.-L.A., unpublished data).

A common property of all of these postulated functions is that there is no obvious need for the sites of action to be at specific, rigidly preserved locations, except for their exclusion from structural genes, where the respective selective pressures during evolution would be unlikely to coincide. This conclusion would be in agreement with the finding that their localization on the chromosome is not conserved between *E. coli* and *S. typhimurium*: most sites identified in one organism are not at an equivalent location in the other organism (40).

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