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Formation of $(dA\text{-}dT)_n$ Cruciforms in *Escherichia coli* Cells under Different Environmental Conditions

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We have detected cruciform formation of $(dA\text{-}dT)_n$ inserts in *Escherichia coli* cells by analyzing the superhelical density of isolated plasmid DNA samples and by probing intracellular DNA with chloroacetaldehyde. The plasmids we used were pUC19 containing inserts of $(dA\text{-}dT)_n$. The cruciforms appeared after cells underwent different stresses: inhibition of protein synthesis, anaerobiosis, and osmotic shock. At the same time, all these stimuli led to an increase in superhelical density of the control pUC19 plasmid DNA. Therefore, we suggest that the increase in plasmid superhelicity in response to different environmental stimuli entails the appearance of cruciform structures. The use of the $(dA\text{-}dT)_n$ units of various lengths made it possible to estimate the superhelical density of the plasmid DNA in vivo.

The existence of cruciforms in superhelical DNA in vitro has been conclusively shown by various methods (6, 7, 19–22, 24, 30, 33), but the existence of cruciforms in vivo is less certain.

Earlier attempts to detect these structures in vivo proved fruitless; neither the natural palindrome in the pAO3 plasmid (23) nor the long artificial palindromes (4, 33) cause cruciforms in the cell. Two possible reasons for the lack of cruciform structures in supercoiled DNA in *Escherichia coli* cells were discussed. First, the actual torsion tension in the cell may be considerably less than the value observed in vitro, making cruciform formation thermodynamically impossible (23, 33). On the other hand, the kinetics of cruciform formation depend dramatically on the length of an inverted repeat (38). It was suggested, therefore, that the formation of cruciforms in long palindromes, occurring as it does at relatively small absolute values of superhelix density, may be kinetically forbidden in vivo; i.e., at physiological temperature the time of cruciform formation may substantially exceed that of an *E. coli* cell generation (4).

Sullivan and Lilley (36) have shown that cruciform relaxation time depends dramatically on the AT content of the sequences flanking the inverted repeat. Thus, one can suppose that long inverted repeats located in the AT-rich regions can form cruciforms in vivo. Indeed, the formation of such cruciforms in *E. coli* cells was detected recently (16, 29).

A study of the structure of $(dA\text{-}dT)_n$ units within supercoiled DNA showed these sequences to adopt the cruciform conformation (10, 13, 31). It is a distinct feature of the $(dA\text{-}dT)_n$ cruciforms that they appear at smaller absolute superhelix densities and have faster relaxation kinetics than any previously studied cruciform structure (31). It seems reasonable, therefore, to expect to find this kind of cruciform in vivo, since both thermodynamic and kinetic barriers to their formation would be less effective. In fact, there have been reports of their presence in *E. coli* cells on incubation with chloramphenicol (13, 28).

This is a detailed study of the mechanism of cruciform formation in vivo for the $(dA\text{-}dT)_n$ inserts in the pUC19 plasmid under various ambient conditions. We have detected cruciform formation in *E. coli* cells by using an indirect approach based on the estimation of superhelicity of isolated DNAs as well as direct chemical probing of the intracellular DNA by chloroacetaldehyde (CAA). The cruciform formation was detected after cells were exposed to different environmental stresses which increase DNA supercoiling: protein synthesis blockage, anaerobiosis, and osmotic shock (5, 14, 23, 25, 40). The use of $(dA\text{-}dT)_n$ inserts of different lengths provided an opportunity to estimate with some accuracy the actual torsion tension of pUC19 plasmid DNA in *E. coli* cells.

MATERIALS AND METHODS

Plasmids. Plasmids pAT18, pAT22, pAT32, and pAT42 carrying $(dA\text{-}dT)_n$ insertions of 18, 22, 32, and 42 bp, respectively, in the pUC19 polylinker have been described earlier (31). The plasmid pA81 containing a $(dA)_{81} \cdot (dT)_{81}$ insertion in the pUC19 polylinker was constructed by adding homopolymeric tails to the *Pst*I site with the calf thymus terminal transferase. The plasmids were maintained in *E. coli* JM83 (41) or C600 (27).

Cultivation of bacteria. Cultures (150 ml) exponentially growing in LB broth at 37°C, reaching an optical density at 600 nm of 0.15 to 0.25, were either immediately fixed by abrupt cooling or further incubated in the presence of 170 μg of chloramphenicol per ml. After different incubation times with the antibiotic, the cells were fixed by rapid cooling.

For experiments involving amino acid deficit, JM83 cells containing the pAT42 plasmid were grown in M9 medium (27) with 0.5% glucose, 1 μg of vitamin B_1 per ml, and 100 μg of proline per ml. On reaching an optical density of 0.15 to 0.25, valine was added up to 1.0 mg/ml, and then the culture was incubated for different times and fixed by abrupt cooling.

Controlled aerobic conditions were achieved by growing C600 cells with plasmid pUC19 or pAT42 in supplemented

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M9 medium with vigorous bubbling of air to an optical density of no higher than 0.2. Semianaerobic conditions were achieved by growing the same strains in supplemented M9 medium without air bubbling to an optical density of 0.6 to 0.7. To create high osmolarity, sodium chloride was added to the medium to a concentration of 0.3 M.

Extraction of plasmid DNA. Plasmid DNA was isolated by the method of Holmes and Quigley (15) with subsequent treatment with RNase, double deproteinization by phenol, and reprecipitation by alcohol.

Gel electrophoresis. Gel electrophoretic separation of DNA topoisomers (32) was performed in 1% agarose (Sigma) in the presence of 5 to 15 μg of chloroquine (Reanal) per ml in a Tris-acetate buffer at 2.5 V/cm for 20 to 24 h with the buffer continually circulated between the two electrode chambers. Gels were then incubated for 1 h in a 2-μg/ml water solution of ethidium bromide, which was followed by 30 min of incubation in distilled water and subsequent restaining with the 2-μg/ml ethidium bromide solution to diffuse out all the chloroquine.

Two-dimensional gel electrophoresis at an ionic strength of up to 0.2 M Na^+ was performed in 1.5% agarose gels as previously described (31). The separation in the first direction was done in sodium phosphate buffer (pH 7.0) of different concentrations with 1 mM EDTA. Because of different ionic strengths the voltage was adjusted so that the temperature of the gel never exceeded 27°C during the run.

Chemical probing of DNA. CAA modification in vitro was performed with 5 μg of pAT32 DNA in 0.2 M NaCl–25 mM Tris (pH 7.5)–2.5 mM EDTA–2% CAA at a final volume of 25 μl. After incubation at 37°C for 30 min, samples were twice precipitated with 3 volumes of ethanol. Modification sites were further analyzed at the sequence level by using primer extension or Maxam-Gilbert DNA sequencing. Primer extension was performed with the Sequenase version 2.0 sequencing kit (United States Biochemical) according to the manufacturer's protocol except that instead of dideoxynucleoside triphosphate termination, 1 μl of deoxynucleoside triphosphate mixture (2.5 mM, each deoxynucleoside triphosphate) was added to the labeling reaction for 5 min at 37°C. For Maxam-Gilbert DNA sequencing, modified DNA samples were treated with formic acid (purine reaction) or hydrazine in high salt (cytosine reaction), which was followed by piperidine treatment.

The in vivo chemical probing was done as follows. *E. coli* JM83 harboring the pAT32 plasmid was grown in L broth containing 100 μg of ampicillin per ml to an optical density at 600 nm of 0.4. Chloramphenicol was then added to a concentration of 170 μg/ml. After 1 h of incubation at 37°C with aeration, 30-ml samples were centrifuged and cells were resuspended in 2 ml of 0.15 M NaCl–50 mM potassium phosphate buffer (pH 7.5). CAA was then added to a final concentration of 2%. After 30 min of incubation at 37°C, cells were diluted 1:10 with the cold buffer and centrifuged. Plasmid DNA was then isolated as described above. Modified DNA bases were detected at the sequence level by using the primer extension technique.

RESULTS

$(dA\text{-}dT)_n$ cruciforms in *E. coli* cells under chloramphenicol treatment. To detect the cruciform formation in vivo, we used two approaches. The first is indirect and is based on the estimation of superhelical density of isolated plasmid DNA. If plasmid DNA in the *E. coli* cell undergoes a structural transition involving the loss of some supercoils, DNA gyrase will tend to compensate for the missing supercoils. Thus, the absolute superhelical density of the plasmid DNA having undergone a structural transition will be higher than that of the same DNA without the transition. One can see this difference after electrophoretic separation of DNA topoisomers. This technique was used earlier for the detection of Z DNA and cruciforms in *E. coli* cells (12, 13, 28).

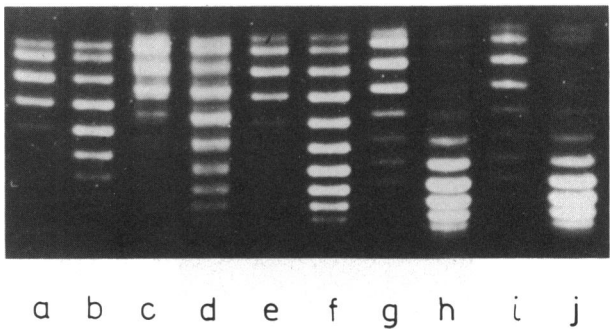

FIG. 1. Electrophoretic separation of DNA topoisomers of pUC19 and pAT plasmids extracted from exponentially growing or chloramphenicol-treated (20 h) *E. coli* cells. Lanes a, c, e, g, and i, Exponentially growing cells; lanes b, d, f, h, and j, chloramphenicol-treated cells; lanes a and b, pUC19; lanes c and d, pAT18; lanes e and f, pAT22; lanes g and h, pAT32; lanes i and j, pAT42.

It has been shown earlier that B-to-Z and B-to-cruciform transitions occur in *E. coli* cells after prolonged amplification of plasmid DNA caused by protein synthesis inhibition by chloramphenicol. Using gel electrophoresis, we examined the patterns of DNA topoisomers for plasmids with different $(dA\text{-}dT)_n$ insertions extracted from exponentially growing *E. coli* cultures or from cultures exposed to chloramphenicol.

The results are presented in Fig. 1. In exponentially growing cultures the patterns of DNA topoisomers for plasmids with insertions and for the vector plasmid pUC19 are not appreciably different (Fig. 1, lanes a, c, e, g, and i). This shows that in the bulk of plasmid DNA in exponentially growing *E. coli* cells the $(dA\text{-}dT)_n$ units do not adopt the cruciform conformation.

A comparison of the topoisomer patterns for the vector plasmid pUC19 extracted from cells of both types shows amplification to have caused a slight shift toward more supercoiled DNA topoisomers (Fig. 1, lanes a and b). The difference between the distribution maxima (ΔLk) is 1.5 to 2 supercoils. Earlier we observed similar changes for the pAO3 plasmid (24). In contrast with this slight shift, amplification of the pAT42 plasmid causes a substantial increase in its superhelicity (Fig. 1, lanes i and j). If we take $\Delta Lk = 1.5$ for the vector plasmid as a correction value, then for pAT42 $\Delta Lk = 4.5$. This value agrees with the loss of supercoils on the transition of the $(dA\text{-}dT)_{21}$ sequence to the cruciform state. Similarly, for pAT32, the corrected value of ΔLk is 3.5 (Fig. 1, lanes g and h).

One can observe a somewhat more involved picture for the plasmid pAT22, which has a relatively short $(dA\text{-}dT)_{11}$ insertion. For plasmid pAT22 the distribution of DNA topoisomers after amplification is much wider than in the exponential culture. This is due to more supercoiled topoisomers, and there is no clear-cut maximum (Fig. 1, lane f). The resultant pattern may be interpreted as a superposition of two topoisomer distributions, because approximately one-half of the intracellular pAT22 DNA molecules have undergone the transition to the cruciform state. Finally, for the

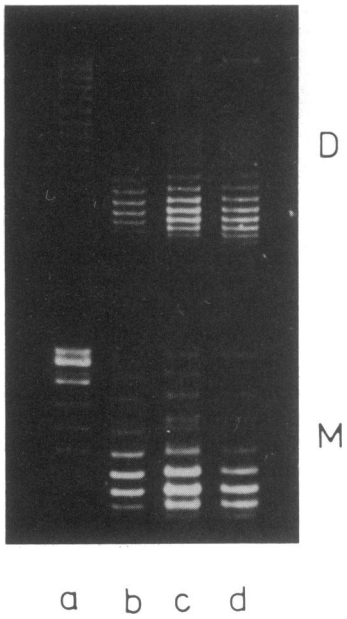

FIG. 2. Electrophoretic separation of DNA topoisomers of pAT42 plasmid extracted from *E. coli* cells treated with chloramphenicol for different times. Lanes: a, untreated cells; b, 0.5 h; c, 1 h; d, 2 h. M, monomeric DNA; D, dimeric DNA.

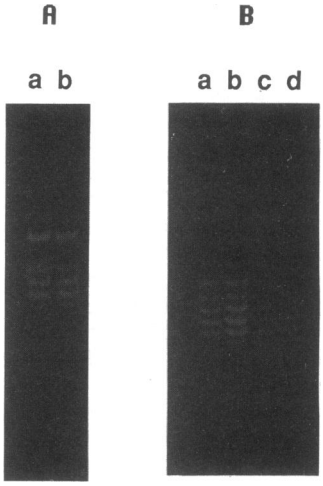

FIG. 3. Electrophoretic separation of DNA topoisomers of pA81, pAT22, and pATM22 plasmids extracted from exponentially growing or chloramphenicol-treated *E. coli* cells. pATM22 plasmid differs from pAT22 by two point substitutions, disrupting the palindromic nature of the insert. (A) pA81. Lane a, Exponentially growing cells; lane b, chloramphenicol-treated cells. (B) pAT22 and pATM22 plasmids. Lanes a and c, pAT22; lanes b and d, pATM22; lanes a and b, exponentially growing cells; lanes c and d, chloramphenicol-treated cells.

plasmid with the shortest insertion, pAT18, the pattern is more blurred than for pAT22 (Fig. 1, lanes c and d).

We also examined the kinetics of the change of supercoiling for the pAT42 plasmid DNA versus time of incubation with chloramphenicol. The previously observed picture, i.e., the sharp increase of supercoiling density in the bulk of plasmid DNA, is already manifest after 0.5 h of incubation with protein synthesis inhibitor (Fig. 2). Within this time the copy number of plasmid DNA does not increase more than twofold (data not shown). Thus, a prolonged amplification of plasmid DNA is not required for the observed effect.

We also should note that in a small number of intracellular DNA molecules of pAT42, though not for plasmids with shorter $(dA\text{-}dT)_n$ insertions, the structural transition takes place in the exponentially growing culture as well (Fig. 1, lane i), causing a few highly supercoiled topoisomers; $(dA\text{-}dT)_n$ insertions of such length begin to be deleted in *E. coli* cells (31).

We have suggested that changes in DNA supercoiling of the plasmids with $(dA\text{-}dT)_n$ insertions are due to cruciform formation followed by DNA gyrase compensatory activity in chloramphenicol-treated *E. coli* cells. Still, we could not rule out the possibility that other structural distortions leading to the same topological consequences, for example, DNA melting, have occurred. To exclude DNA melting, we have performed several additional experiments.

First, we have compared the topoisomer patterns of the plasmid pA81 isolated from *E. coli* cells incubated with or without chloramphenicol. Plasmid pA81 contains an 81-bp $(dA)_n \cdot (dT)_n$ stretch, which is a longer insertion with the same AT content but that is nonpalindromic. In contrast to pAT plasmids, we did not observe any significant difference in superhelical density of plasmid DNAs isolated from both types of cells (Fig. 3A). Second, we have inserted the sequence d(ATATTTATATATATTTTATATAT) into the pUC19 polylinker. This sequence differs from $(dA\text{-}dT)_{11}$ by two A-to-T transversions that disrupt the palindromic character of the insert without changing AT content. One can clearly see the shift towards highly supercoiled topoisomers for pAT22 plasmid DNA isolated from chloramphenicol-treated cells, whereas the topoisomer pattern for the mutant plasmid remains unchanged (Fig. 3B). Thus, we do not see a sharp increase in DNA supercoiling for plasmids containing nonpalindromic AT-rich insertions after exposure to chloramphenicol. Therefore, we believe that in the case of pAT plasmids, the cruciform formation induced by treatment of cells with chloramphenicol is responsible for the observed topological changes.

To prove that this is the case, we used a direct approach based on chemical modification of intracellular plasmid DNA by the chemical carcinogen CAA. This chemical interacts specifically with the base pairing positions of adenine, cytosine, and guanine and is a powerful tool for the detection of non-B DNA conformations such as Z DNA (37) and intramolecular triplexes (17). CAA can penetrate into the cells, making the elucidation of DNA structure in vivo possible (18).

First, we modified the supercoiled pAT32 plasmid in vitro with CAA. After Maxam-Gilbert sequencing of the modified DNA one can clearly see two additional bands in the cytosine ladder that correspond to piperidine cleavage of modified adenines (Fig. 4A). The modified residues were located exactly in the middle of the insert, which corresponds to the looped-out bases in cruciforms. Using a primer extension reaction for the same DNA, we also detected modified bases in the middle of the insert (Fig. 4B). In this case, however, chain breakages were located opposite central adenines as well as thymines. This could be due to DNA polymerase stops at and/or in front of modified adenines.

For in vivo chemical modification of plasmid DNA, cells with plasmid pAT32 incubated with or without chloramphenicol were treated with CAA as described in Materials and Methods. Plasmid DNA was then isolated, and modified bases were detected at the sequence level by primer exten-

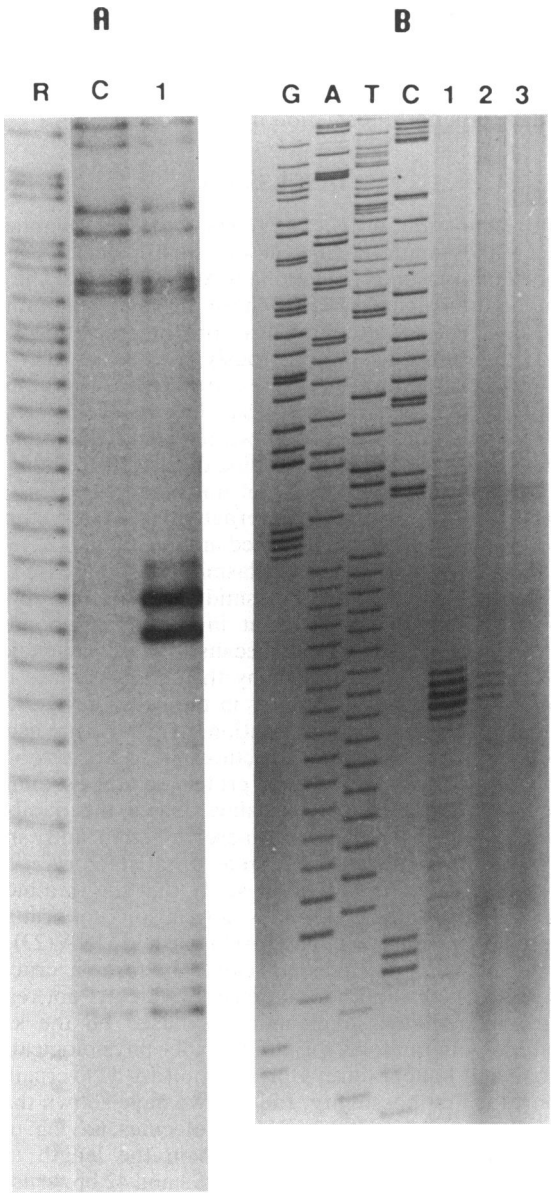

FIG. 4. Patterns of pAT32 modification with CAA in vitro and in vivo. (A) Detection of modified bases by the Maxam-Gilbert technique. Lanes R and C, Standard purine and cytosine ladders, respectively, of unmodified DNA; lane 1, cytosine ladder of CAA-modified DNA in vitro. (B) Detection of modified bases by primer extension technique. Lanes G, A, T, and C, Standard sequencing ladders; lane 1, CAA modification in vitro; lane 2, CAA modification in chloramphenicol-treated cells; lane 3, CAA modification in exponentially growing cells.

sion sequencing (Fig. 4B). DNA polymerase stop sites for pAT32 modified in chloramphenicol-treated cells coincide exactly with those observed in vitro; the stop sites are located in the middle of the $(dA\text{-}dT)_{16}$ insert. For the same plasmid from exponentially growing cells we did not detect any modification in vivo. These data directly show the formation of $(dA\text{-}dT)_n$ cruciforms in *E. coli* cells under chloramphenicol treatment. It is important to note that there is a strong correlation between the topoisomer assay data and the chemical probing data. Thus, we believe that the topoisomer assay is suitable for the detection of cruciform formation in vivo.

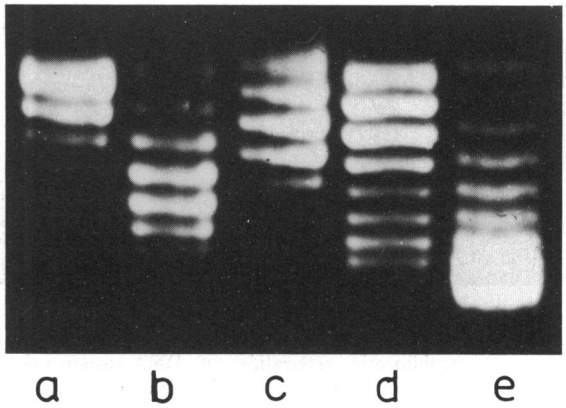

FIG. 5. Electrophoretic separation of DNA topoisomers of pUC19 and pAT42 plasmids extracted from *E. coli* cells under protein synthesis blockage. Lanes a, b, and c, pUC19; lanes d and e, pAT42; lane a, exponentially growing cells; lanes b and e, chloramphenicol-treated cells; lanes c and d, valine-treated cells.

Cruciforms in *E. coli* cells under different environmental stresses. To check whether the observed cruciform formation is the result of a specific effect of chloramphenicol, we examined another case of protein synthesis repression: amino acid deficit. Valine (0.5 to 1 mg/ml) was added to exponentially growing cells containing plasmid pAT42 or pUC19. This situation is conducive to isoleucine deficit, which entails the cessation of protein synthesis and a cell division block (9).

Figure 5 shows the results of electrophoretic separation of pUC19 and pAT42 DNA topoisomers. For the pUC19 plasmid, amino acid starvation leads to an increased superhelical density in a portion of plasmid DNA (Fig. 5, lane c), whereas chloramphenicol shifts the DNA topoisomer distribution as a whole (Fig. 5, lane b). For the pAT42 plasmid, amino acid deficit leads to the appearance of a bimodal distribution of DNA topoisomers, with the additional mode shifted to the area of higher absolute supercoiling by four or five supercoils (Fig. 5, lane d). This corresponds to the formation of cruciforms in a small number of the DNA molecules, whereas chloramphenicol induces the structural transition in an overwhelming majority of the DNA molecules (Fig. 5, lane e).

Figure 6 shows the electrophoretic patterns for pAT42 DNA extracted from cells after different times of incubation with valine. After 1 h, the characteristic distribution (in which the more supercoiled region corresponds to molecules having undergone the structural transition), which remains unchanged during further incubation, is observed. Thus, different ways of inhibiting protein synthesis qualitatively lead to the same result: an early appearance of plasmid DNA molecules with $(dA\text{-}dT)_n$ cruciforms in the cell. The difference between the effects of chloramphenicol and amino acid starvation lies in the proportion of molecules undergoing the structural transition.

One possible explanation for the appearance of $(dA\text{-}dT)_n$ cruciforms under protein synthesis blockage is that this action leads to an increase in the absolute value of negative DNA supercoiling, thus provoking formation of cruciforms. To check this possibility, we used two other types of environmental stimuli: anaerobiosis and osmotic stress.

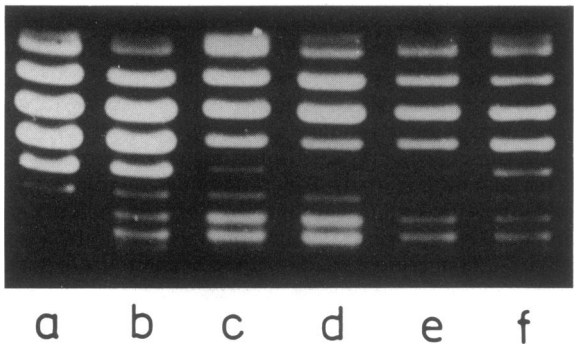

FIG. 6. Electrophoretic separation of DNA topoisomers of pAT42 DNA extracted from *E. coli* cells treated with valine for different times. Lane a, Exponentially growing cells; lanes b through f, cells treated with valine for 0.5, 1, 2, 4, and 8 h, respectively.

These differ dramatically in molecular mechanisms and physiological consequences but converge in their influence on intracellular DNA topology; both increase DNA supercoiling (5, 14, 25, 40).

We cultivated C600 cells containing plasmid pUC19 or pAT42 in a synthetic medium with extensive aeration or without aeration (semianaerobic conditions). The addition of up to 0.3 M sodium chloride provided an osmotic stress. The topoisomer patterns for the DNA isolated from cells under these conditions are presented in Fig. 7. In full agreement with the data published earlier, anaerobic and osmotic stress lead to some increase in pUC19 DNA supercoiling. Anaerobiosis (Fig. 7, lane b) and osmotic stress (Fig. 7, lane c) increase DNA supercoiling only slightly. The strongest effect is observed under a combination of both factors (Fig. 7, lane d).

The separation patterns of DNA topoisomers of the pAT42 plasmid resemble, in part, the data obtained earlier for the case of amino acid starvation. Additional highly supercoiled topoisomers, which could be interpreted as a result of cruciform formation in vivo followed by compensation of missing supercoils with DNA gyrase, can easily be seen. There is also a clear-cut correlation between the extent of the increase in pUC19 DNA supercoiling and the portion of pAT42 DNA molecules undergoing structural transition. A small effect on pAT42 DNA is observed under anaerobiosis or osmotic stress (Fig. 7, lanes f and g), whereas a pronounced effect is observed under simultaneous action of anaerobiosis and high salt (Fig. 7, lane h). Thus, different ways of increasing intracellular DNA supercoiling promote $(dA\text{-}dT)_n$ cruciform formation in *E. coli* cells.

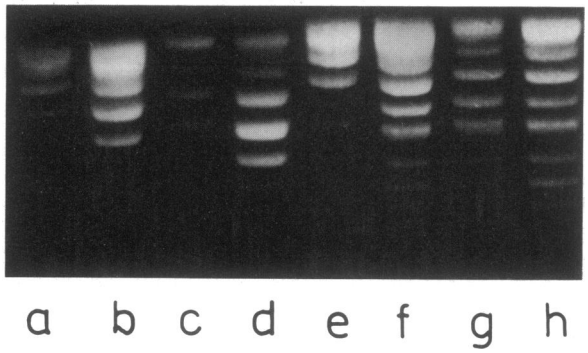

FIG. 7. Electrophoretic separation of DNA topoisomers of pUC19 and pAT42 plasmids from *E. coli* cells under semianaerobiosis and/or osmotic stress. Lanes a through d, pUC19; lanes e through h, pAT42; lanes a and e, aerobic conditions; lanes b and f, semianaerobiosis; lanes c and g, osmotic stress; lanes d and h, semianaerobiosis plus osmotic stress.

DISCUSSION

The incubation of *E. coli* cells with chloramphenicol induces a dramatic change in the superhelical density of intracellular plasmids containing $(dA\text{-}dT)_n$ inserts. The number of additional supercoils in these plasmids quantitatively corresponds to supercoiling loss on the transition of the inserts to cruciforms. As previously shown, $(dA\text{-}dT)_n$ units in vitro form cruciform structures (10, 13, 31). Therefore, we interpreted our data on the supercoiling change in vivo as a result of cruciform formation within intracellular plasmid DNA. The direct chemical probing of the intracellular plasmid DNA with CAA proved that this is the case.

Earlier, the formation of alternative DNA structures in vivo was observed after prolonged amplification (12, 13, 28), when the number of copies of plasmid DNA increased 50 to 100 times for a ColE1-type plasmid (3). Haniford and Pulleyblank (12) hypothesized that in this situation unusual DNA structures might form because of the deficiency of some specific proteins caused by the sharp increase in the number of plasmid DNA copies in the cell. Our data show that the characteristic distribution of DNA topoisomers associated with cruciform structures becomes established after 1 h of incubation with a protein synthesis inhibitor, such as chloramphenicol or valine. Since the number of plasmid DNA copies does not increase significantly in 1 h, we regard the above explanation as unsatisfactory.

We have shown in an earlier study that the treatment of cells with chloramphenicol does not cause cruciform structures to appear in intracellular pAO3 plasmid DNA (23). This may be because this plasmid can only form a cruciform structure at high superhelical density, which is not realistic in the cell. Another explanation is based on the kinetic prohibition of cruciform formation under physiological ionic strength and temperature. Our data on $(dA\text{-}dT)_n$ cruciforms support the first possibility. Indeed, we have shown that the proportion of intracellular DNA molecules having undergone structural transition depends on the length of the $(dA\text{-}dT)_n$ insertion. For insertions of 32 and 42 bp, cruciform structures appear in practically all the molecules, whereas for the 22-bp insertion the transition occurs in only part of the molecules. Since these units are characterized by rapid cruciform formation kinetics, our results clearly show that thermodynamic prohibition holds the greatest significance for the formation of $(dA\text{-}dT)_n$ cruciforms in vivo.

The view that the real DNA superhelical density in the cell is the main limiting factor for the formation of $(dA\text{-}dT)_n$ cruciforms in vivo agrees well with our data on cruciform formation under different environmental conditions. There is a clear-cut correlation between the extent of the increase in pUC19 DNA supercoiling in response to physiologically different stimuli (protein synthesis inhibition, anaerobiosis, and osmotic stress) and the fraction of pAT42 molecules that form cruciform structures under the same conditions.

The data on pAT22 DNA supercoiling in chloramphenicol-treated cells suggest that the $(dA\text{-}dT)_n$ insert in approximately one-half of the intracellular pAT22 DNA molecules undergoes the transition to the cruciform state. Thus, the actual torsion tension of DNA in chloramphenicol-treated cells is comparable to the superhelical density (σ_{tr}) required

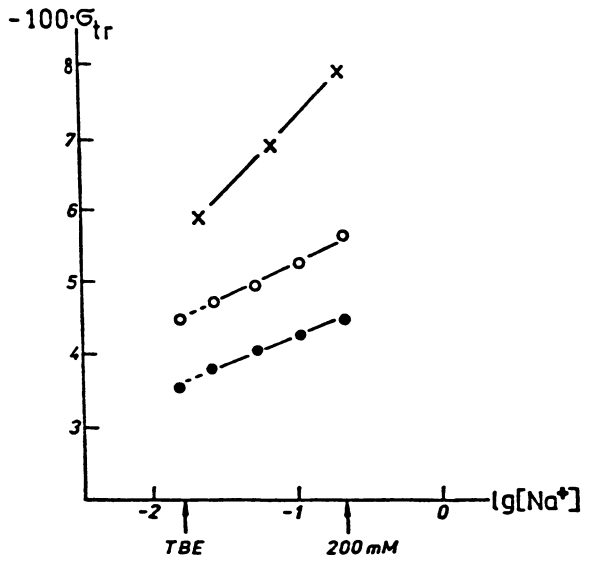

FIG. 8. Dependence of σ_{tr} for pAT22, pAT42, and pAO3 cruciforms on ionic strength. The values of σ_{tr} were obtained by two-dimensional gel electrophoresis performed as described in Materials and Methods. Symbols: ×, pAO3; ○, pAT42; ●, pAT22.

for cruciform formation in the pAT22 plasmid. The data on two-dimensional gel electrophoresis show that at physiological ionic strength (200 mM Na^+), σ_{tr} is −0.057 (Fig. 8). If this value is correct, cruciform formation in pAO3 plasmid DNA under the same conditions would be thermodynamically forbidden (for the cruciform in pAO3, σ_{tr} is −0.079 [Fig. 8]). Similarly, the actual superhelical density of the plasmid DNA inside the cells growing under the combination of anaerobiosis and high salt is very close to the σ_{tr} of the cruciform in pAT42, which is equal to −0.045 (Fig. 8).

It is also important that for the pAT42 plasmid a small number of the DNA molecules undergo the structural transition in cells growing exponentially in rich medium. As mentioned above, the σ_{tr} value for such a cruciform is −0.045 at physiological ionic strength; the torsion tension of plasmid DNA in exponential cultures of *E. coli* can therefore be close to this value.

It is interesting to compare our value of superhelical density in vivo with previously reported data. Study of the binding of psoralen to intracellular *E. coli* DNA led to the value −0.05 ± 0.01 (34), which is close to that which we obtained. Boroviec and Gralla (2) used an indirect method to estimate the actual DNA supercoiling on the basis of comparisons of the strength of the *lacP*^{s} and *lacUV5* promoters in vivo and in vitro and obtained a similar value, −0.048 ± 0.005.

On the other hand, some data which suggest a considerably smaller absolute value (approximately 0.025) of actual DNA tension have been published recently. Bliska and Cozzarelli (1) developed the system of Int-dependent recombination in vivo and estimated the torsion in the cell by the complexity of catenated recombination products; in this case the underestimation may arise because two assumptions made by the authors may not be entirely correct. The authors assumed that (i) DNA in the cell is in the form of a plectonemic superhelix and that (ii) the torsional tension of DNA in the cell can be assessed by the torsional tension of DNA in the region between two recombining sites in a synaptic complex. Zacharias et al. (42) used an approach that is very close to ours and is based on comparison of the B-to-Z transition for the $(dG\text{-}dC)_n$ inserts of various lengths in *E. coli* cells with the transition in vitro estimated by two-dimensional gel electrophoresis. Unfortunately, the latter estimates were made with Tris-borate buffer. We have shown that with 90 mM Tris in the buffer, the B-to-cruciform transitions in pAT22 and pAT42 occur at a lower absolute superhelical density than in 25 mM sodium phosphate buffer (Fig. 8). Thus, we assume that our conditions (200 mM sodium phosphate) are closer to the physiological situation. This also may have been the reason for the underestimation of actual DNA supercoiling in chloramphenicol-treated *E. coli* cells (−0.035) by Sinden and Kochel (35).

It is also difficult to exclude the possibility that the differences between the data are due to the differences in the transcriptional organization of different plasmids. Indeed, it was shown recently that in vivo DNA supercoiling depends dramatically on the transcriptional organization of the plasmid (39).

Our results suggest the following conclusions. The actual superhelical density of pUC19 plasmid DNA in *E. coli* cells exponentially growing in a rich medium is about −0.045. $(dA\text{-}dT)_n$ cruciforms, which may be formed at this superhelical density, appear and are deleted, apparently by enzymes of the recombination apparatus (10). The exposure of cells to some environmental stresses leads to an increase in plasmid DNA supercoiling, which provokes cruciform formation. The factors causing a change in plasmid DNA topology under these conditions are unclear and need further elucidation.

Sequences of the $(dA\text{-}dT)_n$ type are widespread in natural DNA (26). The fact that such sequences can adopt the cruciform conformation in vivo may have extensive implications with respect to the biological role of cruciform structures.

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