Changes in the Linking Number of Supercoiled DNA Accompany Growth Transitions in *Escherichia coli*

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The supercoiling levels of plasmid DNA were determined from *Escherichia coli* which was grown in ways that are known to alter global patterns of gene expression and metabolism. Changes in DNA supercoiling were shown to occur during several types of these nutrient upshifts and downshifts. The most dramatic change in supercoiling was seen in starved cells, in which two populations of differentially relaxed plasmids were shown to coexist. Thus, some changes in the external nutritional environment that cause the cells to reorganize their global metabolism also cause accompanying changes in DNA supercoiling. Results of experiments with dinitrophenol suggested that the observed relaxations were probably not due to reduced pools of ATP. When rifampin was used to release supercoils restrained by RNA polymerase, the cellular topoisomerases responded by removing these new, unrestrained supercoils. We interpret these results as implying that the cellular topological machinery maintains a constant superhelical energy in the DNA except during certain growth transitions, when changes in metabolism and gene expression are accompanied by changes in DNA supercoiling.

The negative supercoiling of DNA is essential to the maintenance of normal cell function, especially in such cellular processes as transcription, replication, and recombination (for reviews, see reference 7 and 38). Several *Escherichia coli* proteins exist that have the ability to alter the level of supercoiling by breaking and rejoining DNA (for a review, see reference 41). Presently, the known enzymes are DNA gyrase (11), which can use ATP to increase negative supercoiling, and topoisomerases I (40) and III (4), which can relax negatively supercoiled DNA. Since supercoiling in vivo is maintained by the opposing actions of these enzymes (5, 29, 30), their relative activities could be regulated to produce various levels of supercoiling. If such a change in supercoiling were to occur in response to physiological stimuli, it could contribute to the global regulation of cellular processes. In this study we investigated the extent to which the level of DNA supercoiling varies during growth of *E. coli*, with emphasis on how these changes could be involved in the global regulation of transcription.

While the importance of DNA supercoiling in the maintenance of cellular processes has been established, changes in DNA supercoiling which could account for global regulatory responses remain to be demonstrated. There are several examples in which plasmids isolated from bacteria grown under different conditions have been reported to vary in the level of supercoiling, including cells grown at different temperatures (12) and cells grown to the stationary phase (34). It has been shown in additional studies (27, 28) that the level of supercoiling of plasmids has some dependence on the local DNA sequence. One special case of control has been reported in which the level of chromosomal supercoiling differed in *Salmonella typhimurium* growing aerobically and anaerobically because of changes in the topoisomerase activities (42).

In studies in vivo with temperature-sensitive gyrase mutants (39) and antibiotics (31, 33, 36), it has been shown that

promoters respond differently to DNA relaxation. In vitro transcription studies have further shown that even small changes in supercoiling can produce very significant changes in the activity of a promoter (1–3). The promoters for gyrase and topoisomerase I have transcriptional activities that are responsive to supercoiling (19, 37). This regulation may homeostatically control the cellular levels of supercoiling and thus prevent significant changes from occurring. As suggested by the results of these various studies on promoter sensitivities, however, even small changes in supercoiling in response to external stimuli could play a significant role in regulating transcription.

In this report we present the results of systematic studies on the effects of changes in bacterial growth conditions on the supercoiling levels of plasmid DNA in transformed cells. We found that significant changes in supercoiling occur when *E. coli* adjusts its metabolism in response to shifts in the available nutrients.

MATERIALS AND METHODS

Bacterial strains and plasmid. *E. coli* MM294 transformed with pBR322 was used unless indicated otherwise. Strain XAC [$\Delta lac pro argE(Am) Nal^r Rif^r ara thi$] was a gift from J. Miller.

Growth conditions. Cells were grown in MOPS (morpholine propanesulfonic acid)-buffered medium (23) supplemented with 1.3 mM potassium phosphate–1 μ g of thiamine per ml–100 μ g of ampicillin per ml. Casamino Acids (Difco Laboratories, Detroit, Mich.) were added at 5 mg/ml, and sugars were added at either 0.2 or 0.5% (wt/vol), unless specified otherwise. Cells were grown at 37°C with vigorous shaking. Cell density was measured at 660 nm with a spectrometer (Sequoia-Turner). In experiments involving antibiotics, the cells were incubated with either 300 μ g of rifampin (Sigma Chemical Co., St. Louis, Mo.) per ml or 150 μ g of chloramphenicol (Sigma) per ml. 2,4-Dinitrophenol (DNP; Aldrich Chemical Co., Inc., Milwaukee, Wis.) was

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recrystallized before use and made in a 0.5 M solution with dimethyl sulfoxide.

Plasmid isolation and electrophoresis. Exponentially growing cells (15 to 25 ml) were rapidly chilled in an ice water bath and collected by a 5-min centrifugation (5,000 rpm) at 4°C. Cells were lysed by the rapid boiling method described by Holmes and Quigley (14). The lysate was treated with RNase A (100 µg/ml) for 30 min at 40°C, followed by proteinase K treatment (100 µg/ml) for 2 to 4 h at 56°C. The DNA was precipitated by the addition of 0.1 volume of 8 M ammonium acetate and an equal volume of isopropanol. The plasmid was suspended in 500 µl of 10 mM Tris hydrochloride (pH 8.0)–1 mM EDTA and extracted once with an equal volume of neutralized phenol. The aqueous phase was extracted repeatedly with an equal volume of phenol–chloroform–isoamyl alcohol (25:24:1) until the interface was clear, after which the DNA was precipitated with ethanol.

Agarose gels (1.4%, 20 by 20 by 0.3 cm) were run horizontally in Tris phosphate buffer (34) with 7 to 10 µg of chloroquine phosphate (Sigma) per ml for 15 to 20 h at 70 to 100 V. After being stained for 2 h in 1 µg of ethidium bromide per ml, the gels were photographed with Tri X film (Eastman Kodak Co., Rochester, N.Y.) under UV illumination. A soft laser scanning densitometer (Zeineh, Fullerton, Calif.) was used to make densitometric tracings from the negatives. The linking number difference representing the center of distribution of the topoisomers was determined from these tracings. To identify the absolute value of the linking number difference, standards were run in parallel and analyzed by the method described by Keller (16). The calf thymus topoisomerase I that was used to make these topoisomer standards was kindly provided by J. Borowiec.

RESULTS

There are many examples of bacterial regulatory networks, such as catabolite repression, growth control, and anaerobic growth, in which numerous promoters are affected simultaneously (13). The transcriptional control mechanisms are mediated in part by changing concentrations of metabolites that are induced by changing external nutrient conditions. In many cases the key aspects of the transcriptional response are uncertain. Studies on promoter responses to supercoiling have suggested a potential role for DNA supercoiling in all of these networks (25, 31, 42). Here we have reported the effects of several types of growth conditions and nutrient transitions on the supercoiling level of a plasmid. The relationship between supercoiling and growth control was investigated by isolating a plasmid from cells that were grown in various media. In addition, the level of plasmid supercoiling was followed during the course of nutrient starvations and upshifts. Finally, to probe the mechanisms that control levels of supercoiling, we looked at the effect of the energy uncoupler DNP and rifampin on intracellular supercoiling levels.

pBR322 was used as a test plasmid for sensing global changes in the cellular machinery that maintains the topological state of *E. coli* DNA. Since pBR322 does not contain promoters that are individually regulated by changes in specific nutrients, only global effects on supercoiling should lead to changes in plasmid supercoiling. By isolating plasmid DNA from cultures of *E. coli* and separating topoisomers on agarose gels, the linking number difference (number of supercoils) could be quantitated. In all of the gels presented in this report, the plasmid was negatively supercoiled; and in a single lane, adjacent DNA bands differed by one supercoil, corresponding to a linking number difference of 1.

TABLE 1. Effect of media composition on supercoiling levels of pBR322

Medium			Doubling time (min)	Linking no. difference ^c
Buffer ^a	Carbon source	CAA ^b		
LB	Included	Included	30	–29.2
MOPS	0.2% Glucose	+	40	–28.5
MOPS	0.2% Glucose	–	50	–27.9
MOPS	0.5% Glycerol	+	40	–26.5
MOPS	0.5% Glycerol	–	80	–26.8
MOPS	0.5% Sodium acetate	+	60	–25.5
MOPS	0.5% Sodium acetate	–	300	–29.0

^a MOPS buffers were supplemented as described in the text.

^b CAA, Casamino Acids at 5 mg/ml.

^c Determined as described in the text. Values are averages of three determinations.

Effect of growth rate on supercoiling levels. In the first set of experiments, the possible role of changing levels of supercoiling in growth control was investigated by isolating pBR322 from cells that were dividing at different rates. Under various growth conditions, a difference is observed in the ratio of stable RNA to mRNA synthesized (24). In slower-growing cells, transcription from rRNA promoters is significantly decreased relative to that in fast-growing cells. The molecular basis of this growth control has not yet been fully established. Results of experiments with gyrase inhibitors suggest that the rRNA promoters are more sensitive than other promoters to changes in DNA linking number (25). This raises the possibility that growth control is due in part to systematic differences in supercoiling levels in response to different growth conditions.

The plasmid was isolated from exponentially growing cells, with doubling times ranging from 30 min to 5 h, a range over which there are substantial changes in the ratio of rRNA to mRNA transcription (24). LB (20) and MOPS media, which were supplemented with a variety of carbon sources and Casamino Acids, were used. The plasmid topoisomers were separated on gels, and the average linking number difference was calculated from densitometer tracings. The linking number differences of the isolated plasmid are listed in Table 1, along with the doubling times of *E. coli* in each medium. Although the number of supercoils in the plasmid isolated from one medium may have varied by up to two linking numbers between experiments, the absolute differences in linking number between the different media were very reproducible. The linking number differences did not appear to correlate with the growth rate (Table 1). Thus, despite the fact that there are enormous changes in the ratio of rRNA to mRNA when slow- and fast-growing cells are compared, there was little or no change in the linking number difference of the test plasmid. This was demonstrated most convincingly by the acetate to LB comparison (Table 1); here the growth rate differed by an order of magnitude, yet the linking number difference was virtually the same.

To investigate the effect of growth phase on supercoiling levels, cells were allowed to grow to a high cell density and enter into the stationary phase for several hours. The same media were used as those described above for the exponential-growth-phase experiments. The isolated plasmid invariably was found to have reduced linking number differences (data not shown), and the extent of relaxation usually varied from two to five supercoils. In general, the relaxation was greater when the cells were grown under conditions that supported higher cell densities before growth ceased. Relax-

ation by as many as 10 supercoils was seen when cells were grown in the rich medium LB.

Effect of removal of essential nutrients. Another type of global control occurs when growing cells are shifted into medium that lacks essential nutrients or that contains poorer nutrients. Under such stress the cells change their global pattern of gene expression, depressing the synthesis of ribosomal components and altering the spectrum of mRNAs (15). Since many operons are affected simultaneously, a global effector may be involved. Changes in DNA supercoiling, which can both increase and decrease transcription, depending on the promoter, could be such an effector. The effects of several different growth shifts on plasmid supercoiling levels therefore were determined.

The plasmid isolated from cells before and after an extreme downshift is shown in Fig. 1. In this downshift, cells growing on glucose and Casamino Acids were collected by centrifugation and rapidly suspended into MOPS medium containing no nutrients. Lane 1 shows the topoisomer distribution of the plasmid isolated from the exponentially growing cells prior to the downshift. In lanes 2 to 4, a time course after the downshift is shown. By 4 h after the downshift (lane 4), the plasmid became relaxed by six supercoils to a new linking number difference of -23. Several control experiments were done to show that the change in supercoiling was not due to the stress of centrifugation at 4°C (data not shown). The loss of DNA supercoiling could be reversed if the downshift was interrupted. Lane 5 shows the plasmid isolated from cells that were downshifted for 2 h and then to which nutrients were added back to restore the original environment. By comparing lane 5 with lane 3 (cells starved for 2 h), it can be seen that the plasmid recovered its supercoils under these circumstances (see also Fig. 6).

Another experiment was done that was similar to that shown in Fig. 1, except that cells were downshifted in a way which allowed for a more gradual adjustment to the poorer nutritional environment. In this experiment cells were grown in medium with only enough glucose present to support growth to the mid-log phase, after which the cells stopped growing. The growth curve of such an experiment is shown in Fig. 2A, along with the linking number difference of the

FIG. 1. Agarose gel electrophoresis of pBR322 isolated from cells after removal of nutrients. Strain MM294(pBR322) was grown in MOPS medium supplemented with 0.2% glucose-5 mg of Casamino Acids per ml. When the cells reached the mid-log phase, they were collected by centrifugation at 4°C and suspended into an equal volume of MOPS medium lacking nutrients. The plasmid was isolated and electrophoresed as described in the text. The plasmid was isolated from cells before they were shifted (lane 1) and after they were downshifted for 1, 2, and 4 h (lanes 2 to 4, respectively). Lane 5 contained plasmid isolated from cells that were starved for 2 h, and then to which 0.2% glucose and 5 mg of Casamino Acids per ml were added back for 30 min. The gel contained 10 μ g of chloroquine per ml.

FIG. 2. Effect of glucose depletion on supercoiling of the plasmid. Strain MM294(pBR322) was grown in MOPS supplemented with 0.05% glucose. At time zero the cells were diluted into the growth medium, and at the indicated times plasmid was isolated and the linking number difference was determined as described in the text. (A) Growth curve of cells showing the linking number difference of the isolated plasmid. OD₆₆₀, Optical density at 660 nm. (B) Agarose gel electrophoresis of pBR322, with the lane numbers corresponding to the sample numbers in panel A. The gel contained 10 μ g of chloroquine per ml.

plasmid isolated at various time points. Representative lanes from the gel corresponding to some of the points in Fig. 2A are shown in Fig. 2B. By comparing Fig. 2B, lanes 1 and 2, it can be seen that as the cells stopped dividing, the plasmid topoisomer distribution became transiently narrowed toward the more supercoiled end of the distribution. As the length of starvation increased, the plasmid became progressively relaxed, as was observed in the analogous, more rapid downshift (Fig. 1).

In the type of severe nutrient downshifts done in these experiments, there were global changes in the pattern of cellular gene expression. These results indicate that such changes, which are largely reductions in expression, are accompanied by reduced levels of DNA supercoiling. When nutrients were added back, expression was increased, and these results indicate that increases in DNA supercoiling also occur. These observations are correlative, thus allowing for the possibility that reduced supercoiling contributes to reduced transcription during downshifts.

Effect of a gradual shift in nutrients. *E. coli* is also known to reorganize its pattern of gene expression after it depletes a preferred carbon source and readjusts to growth in a poorer media. To investigate whether changes in DNA supercoiling accompany these shifts, cells were again grown with a limiting amount of glucose, but the poor carbon source acetate was added. This should have forced the cells to reorganize their metabolism to grow on acetate after the

FIG. 3. Effect of nutrient shift on plasmid supercoiling. Strain MM294(pBR322) was grown in MOPS supplemented with 0.05% glucose–0.5% sodium acetate. At the indicated times, the plasmid was isolated and the linking number difference was determined as described in the text. The time when the cells began growing again on acetate is indicated with an arrow. OD₆₆₀, Optical density at 660 nm.

glucose was depleted. The growth curve of this energy source shift is shown in Fig. 3, along with the linking number difference of the plasmid. Initially, the cells used glucose to grow rapidly, and plasmids isolated from these cells had the usual linking number difference. After the glucose was depleted, the cells entered into a lag phase which lasted for over 2 h. When cell division stopped, the plasmid became transiently more supercoiled, and as the lag phase continued, the plasmid eventually reached a more relaxed state than that of the initial sample (both phenomena were also observed in the experiments just described). On leaving the lag phase, the cells began doubling again at a new, slower rate that is characteristic of acetate metabolism. At this point, supercoiling began to increase until it reached a new steady-state level that was higher than that when the cells were utilizing glucose.

Together, these two sets of experiments provide a description of what happens to DNA supercoiling when cells run out of externally supplied glucose at a low cell density. If no alternative carbon source is available, the DNA becomes progressively relaxed. If acetate is available as an alternative carbon source, then the cells relax the DNA slightly during a lag phase while they are readjusting to the new external environment. In both cases, when growth resumes, the DNA is resupercoiled. Complex changes in the pattern of gene expression are known to accompany these changes in nutrient environments (15). Although these experiments cannot establish cause and effect, they do raise the possibility that nutrient depletion-induced changes in supercoiling contribute to an altered pattern of global transcription.

Growth on glucose is only one of an unlimited number of possible growth conditions, and growth transitions involving other nutrients could lead to different results. We have observed several situations in which the cessation or severe slowing of growth is not accompanied by decreases in DNA supercoiling. These include phosphate starvation, heat shock, addition of α -methyl glucoside, and depletion of Casamino Acids (when used as the sole carbon source). In most of these cases (data not shown), as growth slowed down a slight increase in the level of negative supercoiling was seen. This was again due to the fact that the plasmid

population became more homogeneous toward the more supercoiled end of the topoisomer distribution, similar to that shown in Fig. 2 and 3. Growth changes are not obligatorily coupled to changes in DNA supercoiling. Therefore, some specialized growth condition is required to signal the cell to adjust its DNA supercoiling, and the transition to slow growth is not a sufficient condition.

Effect of glucose addition to slow-growing cells. When glucose is added to cells that are metabolizing a poorer carbon source, catabolite repression occurs. Following such a shift, there is a severe, transient repression of β -galactosidase transcription before expression adjusts to a steady-state level that is lower than that during growth on the poor carbon source (26). The transient phase of catabolite repression is not well understood, but is thought to involve a change in the cyclic AMP levels in the cell. To investigate the possible relationship between transient repression and change in supercoiling, both β -galactosidase induction from the chromosome and plasmid supercoiling were followed after the addition of glucose to cultures that were growing on acetate.

The linking number difference of pBR322 isolated from cells during an upshift from acetate to glucose is shown in Fig. 4. Within the first few minutes the linking number fluctuated, and by 10 min a relaxation was seen. Analogous fluctuations in gene expression have been reported previously (8, 10, 43). During the following 20 min the plasmid began to recover its original level of supercoiling. This same general trend was also seen when the cells were upshifted with glycerol, lactose, or Casamino Acids (data not shown) rather than with glucose. The extent of the changes and their timing varied with the energy source used, but relaxation followed by resupercoiling was always observed.

The results of β -galactosidase assays done on a parallel culture that was upshifted from acetate to glucose with the addition of the *lac* operon inducer isopropyl- β -D-thiogalac-

FIG. 4. Effect of catabolite repression on β -galactosidase expression and plasmid supercoiling. Strain MM294(pBR322) was grown in MOPS supplemented with 0.5% sodium acetate, to which 0.2% glucose was added when the cells reached the mid-log phase. (A) β -Galactosidase induction during the upshift. When the cells had reached an optical density at 660 nm of 0.3, the culture was divided. To half the cells 5 mM IPTG was added at the same time as the glucose (●); to the other half only IPTG was added (○). β -Galactosidase units were determined by the method described by Miller (20). (B) Linking number difference of the plasmid during the upshift. After removal of the first sample (time zero), glucose was added. Cells were harvested at the indicated times, and the linking number difference of the plasmid was determined.

topyranoside (IPTG) at the same time are shown in Fig. 4A. Relief of transient repression occurred between 10 and 20 min (extrapolated line in Fig. 4A), which corresponded closely to the time required for plasmid relaxation under these conditions (Fig. 4B). We observed this same correspondence between relief of transient repression and DNA relaxation if the cells were grown in glycerol and upshifted with glucose, although both occurred at a slightly later time (data not shown). This suggests that the changes in supercoiling and *lac* expression are coupled in some manner. The addition of cyclic AMP, however, relieved the transient repression of β -galactosidase without affecting the supercoiling changes of the plasmid during the upshift (data not shown). Therefore, while a common factor may be involved in the two phenomena, they are not causally linked.

Effect of prolonged starvation. When cells are subjected to prolonged starvation, they undergo a series of changes to ensure viability until nutrients are available. These include breakdown and reorganization of polyribosomes (6), a change in the rate of protein degradation (21), and the shutdown of most transcription. To investigate the possible role of a change in the DNA topology in transcriptional regulation, the plasmid was isolated from cells after prolonged nutrient starvation. If the cells were starved (Fig. 1 or 2) for extended periods of time, the plasmids became even more relaxed than was shown, and an unusual bimodal topoisomer distribution could be seen. A densitometric tracing of the topoisomer distribution of the plasmid isolated from cells that were starved for 15 h is shown in Fig. 5A, in

FIG. 5. Effect of prolonged starvation on plasmid supercoiling. (A) Densitometric tracing of a negative of an agarose gel of the plasmid isolated from strain MM294(pBR322) that was starved for 15 h, as described in the legend to Fig. 1. The position of the nicked plasmid is indicated with the arrow. (B) Two-dimensional agarose gel electrophoresis of pBR322 isolated from cells that were starved for 18 h. The first dimension (vertical) was run with 3 μ g of chloroquine per ml, and the second dimension (horizontal) was run with 20 μ g of chloroquine per ml.

FIG. 6. Effect of glucose addition to starved cells. Strain MM294(pBR322) was starved for 15 h, as described in the legend to Fig. 2. Plasmid was isolated from these cells (lane 1) and from cells to which 0.2% glucose was added for 2.5 min (lane 2). The plasmid was also isolated from starved cells that were treated with 10 mM DNP for 10 min (lane 3). Lane 4 shows plasmid from DNP-treated cells to which 0.2% glucose was added for 2.5 min. The gel contained 10 μ g of chloroquine per ml.

which two regions of high band intensity can be seen. When plasmid isolated from these cells was run on a two-dimensional gel (Fig. 5B), the double distribution could be seen more clearly. The more negatively supercoiled end of the distribution was centered at a linking number difference of -23 , and the other end was centered at -13 . Although the cells were not doubling during this time, they remained 100% viable, as measured by plating efficiency. Comparison of the results shown in Fig. 5B for cells starved at a low cell density with those reported above for cells in the stationary phase at a high cell density indicate that there appear to be different mechanisms involved in the determination of the final level of supercoiling.

Cells that were starved overnight were capable of restoring the original level of supercoiling, as was also shown for cells that were starved for 2 h (Fig. 1). In this experiment (Fig. 6), glucose was added back to cells that had been starved for 15 h. By comparing lane 1 (preshift) with lane 2, 2.5 min after the upshift, the plasmid no longer had a bimodal distribution and regained its original level of supercoiling. As a control, chloramphenicol, a protein synthesis inhibitor, was added to the cells 15 min before the addition of glucose. This did not prevent rapid recovery (data not shown). Therefore, new protein synthesis is not necessary for the recovery of supercoils, and the relaxation is not due to the degradation of gyrase.

Effect of DNP treatment. Since DNA gyrase has a requirement for ATP, the relaxations observed in these experiments could be attributed to changing ATP pools during the growth shifts. A severe downshift experiment related to that shown in Fig. 1 has been reported previously (17), but the results differed significantly in that it was done in the presence of the energy uncoupler (DNP). In those experiments the plasmid became relaxed, but this was attributed to a decrease in ATP pools, which caused a decrease in gyrase activity. The level of ATP is also known to drop quite rapidly after nutritional upshifts (18). We investigated the possibility that the relaxation of the plasmid that we observed during the downshift (Fig. 1) and during the nutritional upshift (Fig. 4B) was due to decreasing ATP levels.

Plasmid was isolated from cells that were growing in MOPS supplemented with 0.2% glucose–5 mg of Casamino Acids per ml and that were treated for 30 minutes with concentrations of DNP ranging from 0.1 to 10 mM (data not shown). Concentrations below 1 mM DNP caused the plasmid to become slightly more supercoiled. Concentrations

FIG. 7. Effect of rifampin treatment on the linking number difference of the plasmid. Strain MM294(pBR322) was grown in MOPS supplemented with 0.2% glucose–5 mg of Casamino Acids per ml. The plasmid was isolated from untreated cells (lane 1), cells treated with 300 μ g of rifampicin per ml (lane 2), and cells treated with 150 μ g of chloramphenicol per ml (lane 3). Lane 4 contained the plasmid isolated from strain XAC(pBR322) after treatment with 300 μ g of rifampin per ml. Incubations with antibiotics were done for 30 min.

above 1 mM caused the plasmid to become more relaxed by one or two supercoils. The results were the same if the plasmid was isolated anywhere from 5 to 60 min after the addition of DNP. No correlation was seen between the concentration of DNP and the extent of relaxation, even though it has been reported (22) that higher concentrations of DNP cause a greater decrease in ATP levels. The reported decreases in ATP levels caused by the addition of DNP were greater than those that occurred during the first few hours following the upshifts and downshifts (18, 32) used in these experiments. Therefore, the plasmid relaxation seen in these experiments is probably not due to the decrease in ATP levels that could alter the activity of DNA gyrase.

In contrast, results of another experiment showed that the relaxation seen in cells starved overnight was at least in part, due to a low level of ATP. DNP was added to starved cells before they were allowed to recover by the addition of glucose. By comparing lane 4 with lane 2 (Fig. 6), it can be seen that the DNP partially inhibited the plasmid from regaining its supercoils. Therefore, the rapid recovery of supercoils after the addition of glucose may be due to a rapid buildup of ATP, thus leading to an increase in gyrase activity.

Rifampin treatment as a probe of supercoiling control. The results from the experiments described above established that DNA supercoiling can be adjusted *in vivo* in response to the external environment. Changes occurred during growth transitions, and the levels also depended on the type of growth medium that was used. There were no clues, however, regarding how the cell determines the level of supercoiling that is appropriate to each physiological situation. The only known effector of the topoisomerases is ATP, and in the one case discussed above, a change in ATP levels could not account for a change in the DNA linking number. It is possible that the activity of the topoisomerases changes in response to a number of factors in the cell that allow supercoiling to be set to a new level. Alternatively, the topoisomerases may be responding to a change in the number of unrestrained supercoils, those not taken up by DNA-binding proteins. It has been shown that approximately half of the supercoils on plasmids *in vivo* are unrestrained (35). If during nutritional shifts the number of unrestrained supercoils changes as a consequence of a change in the levels of DNA-binding proteins, it is possible that the topoisomerases readjust the linking number to keep

the number of unrestrained supercoils constant. Goldstein and Drlica (12) have shown that the topoisomerases can sense the change in DNA structure caused by a shift in temperature and change the DNA linking number to compensate for it. We devised an experiment to test the related idea that during steady-state growth cells adjust the linking number because the topoisomerases are set to maintain a constant level of unrestrained superhelical energy.

In principle, the treatment of cells with rifampin should lead to a transient increase in the superhelical energy of intracellular DNA. Rifampin prevents the initiation of transcription by RNA polymerase, a protein that is known to be very effective in taking up negative supercoils (9). Rifampin lowers the number of RNA polymerases that are bound to DNA inside the cell, which should lead to a release of negative supercoils and increased superhelical energy. If the topoisomerases were to maintain a constant level of unrestrained superhelical energy during steady-state growth, then a relaxation of the plasmid would be expected to adjust the energy to the appropriate value.

This relaxation was indeed observed (Fig. 7). The plasmid that was isolated from cells before the addition of rifampin is shown in lane 1. In lane 2, the cells were incubated with rifampin, and the plasmid became relaxed by four to six supercoils. Since RNA polymerase can melt approximately 17 base pairs (9), this decrease in supercoils corresponded to the removal of about three polymerases. Rifampin treatment indirectly inhibits protein synthesis, and as a control, the cells were incubated with the direct protein synthesis inhibitor chloramphenicol for the same amount of time. The plasmid isolated from these cells is shown in lane 3. No relaxation was seen, confirming that the relaxation observed with rifampin was due to the release of RNA polymerase. As a further important control, rifampin was added to a rifampin-resistant *E. coli* strain, XAC(pBR322), the mutation for which lies in the RNA polymerase gene. The plasmid did not become relaxed (lane 4), indicating that relaxation is related to the lack of RNA polymerase activity. These results support the hypothesis that the linking number is controlled by the topoisomerases that sense the availability of superhelical energy and that adjust the DNA topology accordingly.

DISCUSSION

The results from these experiments indicate that changes in *E. coli* plasmid supercoiling can occur in response to changes in externally supplied nutrients. The most significant of these changes involved decreases in the number of negative supercoils, or relaxations, of the plasmid. These relaxations occurred principally when the primary nutrient source was changed abruptly and the bacteria had to adjust their metabolism to the new environment (Fig. 1 to 3 and 4B).

Modest increases in the level of negative supercoiling were observed during slowdowns in growth (Fig. 2 and 3). The plasmid population became more homogeneous, tightening up toward the more negatively supercoiled end of the topoisomer distribution (Fig. 2, lane 2). If a shift in nutrients was not involved, e.g., phosphate starvation or the addition of α -methylglucoside (data not shown), then the plasmid remained at a slightly elevated level of supercoiling. If a shift in nutrient metabolism (Fig. 3) or starvation (Fig. 2) followed the growth slowdown, the plasmid became relaxed.

The results of this study are in good accord with those of previous studies and add to the understanding of supercoil-

ing control. During steady-state (exponential) growth, *E. coli* appears to keep a constant superhelical energy in plasmid DNA (Fig. 7) (12). Part of this homeostatic control may be due to the regulatory nature of topoisomerase expression, which has been reported to be controlled in response to supercoiling (19, 37). When cells are growing exponentially, small differences in supercoiling levels are observed between some nutrient conditions (Table 1) and between different growth phases (data not shown) (34). When the external environment changes, global changes in metabolism are accompanied by changes in the topological state of the DNA. These are observed as fluctuations in linking number before the cells adjust the DNA linking number to a new steady-state value (Fig. 3 and 4B).

The magnitude of the observed supercoiling changes during growth transitions is probably large enough to have significant effects on transcription. Since pBR322 normally contains 28 supercoils, a relaxation of four supercoils represents about a 15% change. Approximately half of the plasmid supercoils are restrained in vivo (35), however, making a maximum change of 30% possible if the number of restrained supercoils remains constant. Changes of 15 to 30% have been shown to have significant effects on differential transcription in vitro (1-3). Whether the linking number changes seen in response to external stimuli are actually used in this manner in vivo is a matter that is currently under investigation.

Since changes in linking number are mediated solely by the topoisomerases, they adjust the amount of supercoiling to a new level in response to external stimuli. The supercoiling variations could be due to either direct modulations of topoisomerase enzyme activities or to the topoisomerases that interact with altered DNA templates. Presently, no known factors affecting intrinsic topoisomerase activities have been discovered, with the exception of the DNA gyrase requirement for ATP. Lower levels of ATP in the cell could cause a decrease in gyrase activity. The results presented here with DNP, however, suggest that ATP levels do not play a significant role in the majority of cases studied; ATP was shown to be important only in a specific case in which an increase in ATP levels was necessary for the recovery of supercoils after prolonged starvation (Fig. 6). Thus, the identity of the factors involved in mediating these changes in supercoiling is currently unresolved, but results of two other experiments described above bear importantly on this issue.

The experiments in which reduced supercoiling was shown after rifampin treatment may provide an important clue to understanding the mechanism underlying these changes. Rifampin treatment leads to the removal of elongating RNA polymerase from DNA, causing an increase in the superhelical energy of the DNA. The fact that the plasmids subsequently become relaxed (Fig. 7) suggests that supercoils are removed by the topoisomerases to readjust the available or unrestrained energy to its former, pre-rifampin treatment value. Thus, it may be that under any fixed growth condition the cell strives to maintain DNA templates with a fixed superhelical energy, as is also suggested by results of temperature shift experiments (12).

Another clue comes from the unusual bimodal topoisomer distribution of plasmids isolated from starved cells (Fig. 5). This result suggests that variations in the number of supercoils restrained by DNA-binding proteins may be of importance. Starved cells show two distinct populations of plasmids; one has lost one-quarter of its supercoils and the other has lost one-half. Presumably, both populations interact with the same cellular pools of topoisomerases and

DNA-binding proteins, yet some mechanism exists for maintaining two populations with very different linking numbers. Since one-half of the supercoils are normally restrained by DNA-binding proteins in vivo, the more relaxed plasmids may have been shut down during prolonged starvation by removal of all available, unrestrained supercoils. Since this can be done while supercoils are retained in another population of plasmids, it seems that a mechanism exists for restricting the accessibility of DNA, perhaps analogous to the case of inactive, condensed DNA in eucaryotes.

Overall, we have shown that changes in DNA supercoiling accompany changes in external nutrients, which are conditions that are known to trigger changes in gene expression. These changes in linking number difference indicate a reorganization of the topological machinery; that is, they reflect changes in the interplay between topoisomerases and DNA-binding proteins. However, they are not necessarily indicative of changes in torsional tension, the determination of which requires knowledge of the extent of supercoiling restraint by proteins. In an additional series of experiments (unpublished data), coumermycin was used to monitor changes in the level of unrestrained supercoils during some of the growth transitions presented here. In several cases, principally during downshifts, we found that the modest topological changes observed in the linking number experiments described above reflect much larger changes in DNA torsional tension. The relationship between these large topological changes and the regulation of physiological processes driven by supercoiling is currently under investigation.

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