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In Vivo Supercoiling of Plasmid and Chromosomal DNA in an *Escherichia coli hns* Mutant

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We have used trimethylpsoralen to measure localized levels of unconstrained DNA supercoiling in vivo. The data provide direct evidence that plasmid and chromosomal DNA supercoiling is altered in vivo in an *hns* mutant. This increase in supercoiling is independent of transcription or changes in the activity of topoisomerase I. These data have implications for the mechanisms by which the chromatin-associated protein H-NS may influence chromosome organization and gene expression.

The *Escherichia coli* chromosome is organized as a highly condensed structure, the nucleoid. The two most abundant architectural proteins in the nucleoid are HU and H-NS (7). H-NS is a 16.5-kDa polypeptide which binds DNA relatively nonspecifically, although it exhibits a preference for curved DNA (15, 27, 44). Mutations in the *hns* gene are highly pleiotropic, affecting genome stability (16), recombination-related events (5, 11), and transcription from a variety of promoters (9, 10, 13, 21). Many H-NS-dependent promoters are sensitive to factors which alter DNA supercoiling, and it has been suggested that H-NS may influence transcription through changes in DNA topology (10, 11, 14, 26). Consistent with this hypothesis, H-NS has been shown to constrain DNA supercoils in vitro (42), and *hns* mutants show changes in the linking number of plasmid DNA isolated from cells (10, 12, 13).

Although changes in plasmid linking number provide an indication of the level of DNA supercoiling in vivo, this method of assessment suffers from several limitations. First, linking number reports the level of supercoiling of naked DNA after purification from the cell. Because of the constraining influence of proteins bound to DNA in vivo, changes in linking number do not necessarily correspond to changes in unconstrained supercoiling in vivo (1, 17). Second, linking number can report only the mean level of supercoiling throughout an entire DNA molecule; localized domains of supercoiling have been shown to exist within a plasmid (25, 33, 45). Finally, of course, changes in plasmid linking number cannot be used to determine levels of chromosomal supercoiling (8, 30).

To circumvent these limitations, and to measure plasmid and chromosomal DNA supercoiling directly in vivo, we have used the DNA cross-linking reagent trimethylpsoralen. Psoralen derivatives are able to penetrate into living cells and intercalate into DNA in a supercoiling-dependent fashion (38). Upon irradiation with long-wavelength UV light, psoralen forms covalent cross-links. The rate of formation of these cross-links is proportional to the level of supercoiling of the DNA in the cell (4, 25) and can detect changes in superhelical density of as small as 12% (25). Using this approach, we have shown that *hns* mutants have increased negative supercoiling of plasmid DNA in vivo. Furthermore, we demonstrate that chromosomal supercoiling is similarly altered. These studies provide a direct demonstration that the net level of negative supercoiling of both plasmid and chromosomal DNA in vivo is increased in *hns* mutants. These findings have implications for the mechanisms of H-NS action.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *E. coli* strains used for cross-linking experiments were the *hns*$^+$ strain GM37 [MC4100 Φ(*proU-lacZ*) hyb2 (λ*plac* Mu15)] and its congenic *hns* derivative GM230 [GM37*osmZ205::*Tn*10*] (10). The *topA* strain RED31 (Hfr PO42 *thi-1 rel-1 lac-42 acrA topA20*::Tn*10 toc-1*) (34) was used for certain experiments. Bacteria were grown at 37°C in an orbital shaker (200 strokes min^{-1}) in LB or minimal medium A (23) supplemented with 0.1% Casamino Acids and 0.4% glucose (MMAA), as indicated. Antibiotics were used, when appropriate, at the following concentrations: ampicillin, 50 μg/ml; tetracycline, 12.5 μg/ml; and kanamycin, 25 μg/ml. Cell growth was monitored by measuring the optical density of the culture at 600 nm.

Plasmids and transformation. Plasmid pLEU500Tc was generously provided by D. M. J. Lilley (University of Dundee, Dundee, United Kingdom) and is described in detail elsewhere (2). pLEU500Tc is a derivative of pAT153 with a 199-bp *Eco*RI-*Hin*dIII fragment containing positions −80 to +87 of the *leu-500* promoter (36), inserted between the divergently transcribed *tetA* and *bla* genes. Plasmid pAV375 is a derivative of pBR322 containing a 940-bp fragment (from −207 to +735 [36]) of the *proU* promoter region of *Salmonella typhimurium* (this fragment includes the downstream regulatory element [DRE]) (Fig. 1). Its construction is described in detail elsewhere (27). Plasmid pFM375 is a derivative of pAV375 constructed by excision of the *Eco*RI fragment containing the *proU* promoter region.

Plasmid DNA was isolated by using the Wizard Minipreps DNA purification system (Promega). Cells were transformed by electroporation using a Gene Pulser apparatus (Bio-Rad Laboratories Ltd.).

DNA photo-cross-linking using TMP. To photo-cross-link DNA, cells in logarithmic growth phase were concentrated 20-fold by centrifugation for 5 min at 3,000 × *g* and resuspended in the appropriate volume of M9 salts (23) at 4°C unless indicated otherwise. All subsequent steps were performed in the dark. A solution of 4,5′,8-trimethylpsoralen (TMP; Sigma) in ethanol was added to a final concentration of 0.25 μg/ml and allowed to equilibrate for 5 min. Samples of 0.5 ml in 35- by 10-mm petri dishes were irradiated for various periods of time under long-wavelength UV light (λ_{max} = 366 nm) at an intensity of 0.6 mW/cm^2 delivered by a Mineralight lamp (UVP Inc.). Because TMP photodestructs above about 20 kJ/m^2 (40), additional TMP was added every 10 min to ensure a linear rate of photoaddition. After irradiation, cells were lysed, and total DNA was isolated and purified as described previously (25). Unlinked TMP was eliminated from the solution by three extractions with phenol-chloroform-isoamyl alcohol (25:24:1, vol/vol/vol) followed by extraction with chloroform-isoamyl alcohol (24:1, vol/vol) and precipitation with ethanol.

Gel analysis of cross-linked DNA. After purification, photo-cross-linked DNA was digested with appropriate restriction endonucleases. In the case of plasmids, the DNA was digested with *Eco*RI: pFM375 was linearized, while two fragments, a 970-bp fragment encompassing positions −207 to +735 of the *proU* promoter and a 4.2-kb fragment containing the rest of the plasmid, were generated from pAV375 (Fig. 1). For analysis of the chromosomal maltose operon, DNA was digested with *Xmn*I, isolating a 1.25-kb fragment extending from the 3′-terminal region of *malE* through the *malE-malF* intergenic region and into the 5′-terminal region of *malF* (22). For analysis of the chromosomal enolase gene, DNA was

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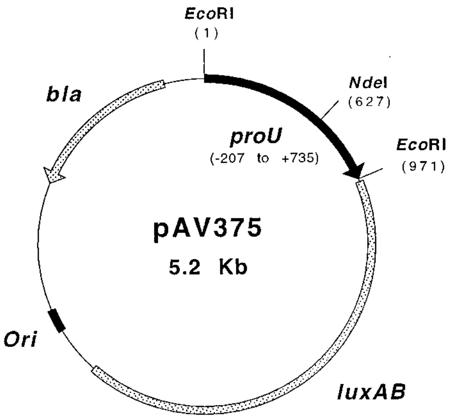

FIG. 1. Map of plasmid pAV375. This plasmid contains a 970-bp *Eco*RI-*Eco*RI fragment encoding positions −207 to +735 of the E. coli *proU* promoter (41). Relevant restriction sites are indicated. pFM375 is pAV375 from which the 970-bp *Eco*RI fragment has been deleted.

digested with *Hae*III, isolating a 950-bp fragment contained within the coding sequence (32). For analysis of the chromosomal *proU* promoter, DNA was digested with *Eco*RV and *Nde*I to isolate a 1,045-bp fragment extending from −630 to +416 around the transcription start site of the *proU* operon (41).

Following digestion, DNA was precipitated with ethanol and suspended in water. One microgram of total DNA was denatured with NaOH and neutralized as described previously (4). Samples were electrophoresed in 0.8% (for the larger fragments) or 1.5% (for the shorter fragments) agarose gels in Tris-borate-EDTA (37) at 2 V/cm until double-stranded and single-stranded DNA fragments of the size of interest were resolved. After electrophoresis, the gels were soaked for 30 min in 0.25 M HCl, followed by 2 h of incubation in denaturing solution (0.5 M NaOH, 1.5 M NaCl). Gels were transferred to Hybond-N$^+$ nylon membranes (Amersham) in 0.4 M NaOH overnight (35). Membranes were hybridized with the appropriated DNA fragments.

DNA probes against plasmids pAV375 and pFM375 were prepared by *Eco*RI digestion of purified pAV375 (Fig. 1). The *malEF* probe consisted of an *Eco*RI-*Bam*HI fragment from plasmid pCH77 encompassing the *malE-malF* intergenic region (22). The enolase probe was a 950-bp *Hae*III-*Hae*III fragment from within the *eno* gene (GenBank accession no. X82400), excised from a pET11a plasmid (32). The chromosomal *proU* promoter probe was a 625-bp *Eco*RI-*Nde*I fragment from pAV375 encompassing positions −210 to +415 (Fig. 1). The DNA fragments to be used as probes were separated on agarose gels, excised and purified by using a Gene Clean kit (Bio 101), and labelled with [α-^{32}P]dCTP (3,000 Ci mmol^{-1}; Amersham International plc.), using the Prime-a-Gene labelling system (Promega). After standard washing, radiactivity was detected by PhosphorImaging or autoradiography using Kodak X-Omat XAR-5 film.

Cross-linking data analysis. Data from the hybridization experiments were analyzed by using a Molecular Dynamics PhosphorImager and ImageQuant software. The level of cross-linking for any DNA fragment was expressed as the amount of double-stranded DNA as a proportion of the total amount of DNA (double stranded plus single stranded). To compare the rates of cross-linking of fragments of different sizes, the data were normalized by dividing by the length of the corresponding fragment. Data are expressed graphically, as the percentage of double-stranded DNA per kilobase as a function of the irradiation time in minutes. This relationship was linear (correlation coefficient higher than 0.99) for all experiments described. The slope of the resulting straight line gives the rate of cross-linking and, hence, provides an estimate of the relative level of unconstrained supercoiling.

Plasmid linking number analysis. The topoisomer distribution of plasmid DNA preparations was analyzed by electrophoresis in 0.8% agarose gels in Tris-borate-EDTA containing chloroquine (2.5 μg/ml). Preliminary studies (not shown) using two-dimensional electrophoresis demonstrated that negative supercoils are detected under these gel conditions. Gels were electrophoresed, stained, and photographed as described previously (24).

RESULTS AND DISCUSSION

Plasmid DNA supercoiling in an *hns* strain. The rates of TMP cross-linking for plasmids pAV375 and pFM375 (pAV375 lacking the *Eco*RI-*Eco*RI *proU* promoter fragment) were estimated in the *hns* strain GM230 and its parental *hns*$^+$ strain GM37. As growth rate and conditions can affect plasmid topoisomer distributions (6, 10, 26), these studies were carried out in media in which differences in growth rate between the *hns* and *hns*$^+$ strains were minimal (in LB and MMAA media, the rate of growth was only 1.1 to 1.2 times higher for the *hns*$^+$ strain than for the *hns* strain [data not shown]). Cultures grown to logarithmic phase were concentrated and incubated with TMP for 5 min (see Materials and Methods). Samples were UV irradiated for the indicated periods of time, either at room temperature (20°C) or at 4°C. After isolation, DNA was digested with *Eco*RI and the rate of TMP cross-linking for each relevant DNA fragment was determined. For plasmid pAV375, *Eco*RI generates two fragments, a 970-bp *proU* promoter fragment and a 4.2-kb vector fragment, which were analyzed separately (Fig. 1). For pFM375, *Eco*RI digestion generates the single 4.2-kb vector fragment.

Initially, the rates of cross-linking of the two fragments of pAV375 were analyzed, with cross-linking carried out at room temperature. For the 4.2-kb vector fragment, a 1.2-fold increase in the rate of cross-linking (negative supercoiling) was observed for the *hns* strain compared with the parental *hns*$^+$ strain (0.93 compared with 0.77; Fig. 2A). For the *proU* promoter fragment, the increase in the rate of cross-linking in the *hns* strain was much greater than for the vector fragment (2.8-fold; 2.63 compared with 0.95 [Fig. 2B]). This could be due to a specific effect of *hns* on the topology of the *proU* promoter fragment, as this fragment contains the known site of action of H-NS (27). Alternatively, because *proU* transcription is derepressed in an *hns* strain (10), transcription itself might induce a change in topology: we have previously shown that transcription can generate local domains of supercoiling detectable by the TMP method (25). When the rate of cross-linking was measured with irradiation carried out at 4°C, which severely reduces transcription rates, the increased rates of cross-linking in the *hns* strain compared with the *hns*$^+$ strain were similar for both the *proU* and vector fragments (Fig. 2C and D). Thus, when any specific effects on *proU* transcription are excluded, the *hns* mutation results in similar increases in the rate of cross-linking (level of negative supercoiling) for both regions of the plasmid.

Once the specific contribution of *proU* transcription is excluded, two important conclusions can be drawn (Fig. 2C and D). First, the rate of cross-linking of the *proU* promoter fragment was reproducibly greater than that of the vector fragment. This result suggests that the absolute level of supercoiling of the *proU* promoter region of the plasmid may be greater than that of the rest of the plasmid, although the possibility that the two fragments have different numbers of TMP-reactive sites cannot be excluded. Second, and most important, the rate of cross-linking of any given fragment was always about 1.4-fold higher in the *hns* strain than in the *hns*$^+$ parental strain (Fig. 2C and D). Sequence differences which might affect the rate of cross-linking are not relevant when one is comparing the same fragment in *hns* and *hns*$^+$ strains. Similar results were observed in LB medium and LB supplemented with glucose (data not shown). Thus, we conclude that the net level of unconstrained negative supercoiling is greater in the *hns* strain than the *hns*$^+$ parent for both regions of the plasmid.

Although we cannot formally exclude the possibility that the altered rates of cross-linking are due to H-NS influencing the accessibility of DNA to TMP, rather than a change in supercoiling, this seems unlikely. The on and off rates of H-NS for DNA are rapid (15), and H-NS does not protect DNA from other agents such as $KMnO_4$ or dimethyl sulfate (data not shown); the rate of cross-linking was the same for the *proU* fragment which is known to contain a site for H-NS action; and

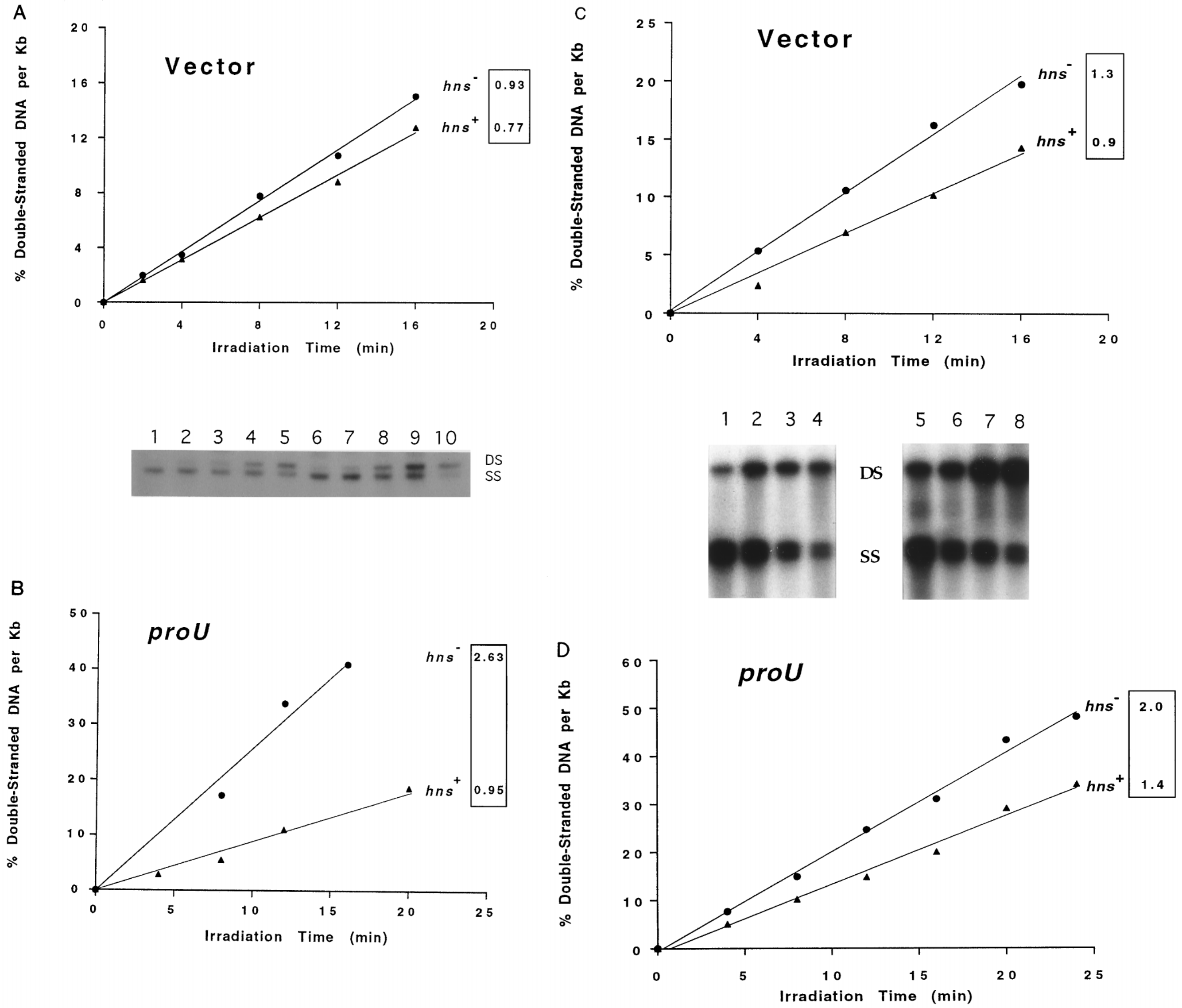

FIG. 2. TMP photo-cross-linking of pAV375 in the hns^+ strain GM37 (triangles) and the *hns* mutant GM230 (circles) grown in LB. The two *Eco*RI-*Eco*RI fragments of pAV375 (vector and *proU*) were analyzed from the same experiments. Irradiation was performed at 20°C (A and B) or at 4°C (C and D). (A) Southern blot analysis of the cross-linked 4.2-kb *Eco*RI-*Eco*RI fragment of pAV375 in the hns^+ (lanes 1 to 5) and *hns* (lanes 6 to 10) strains. Cells harboring plasmid pAV375 in logarithmic phase in LB medium were equilibrated with TMP for 5 min and cross-linked by irradiation at 20°C for 2 (lanes 1 and 6), 4 (lanes 2 and 7), 8 (lanes 3 and 8), 12 (lanes 4 and 9), or 16 (lanes 5 and 10) min. After cross-linking, plasmid DNA was denatured, neutralized, and digested with *Eco*RI. Blots were hybridized with the 4.2-kb *Eco*RI probe from plasmid pAV375. PhosphorImager quantitation of data is presented. (B) Quantitation of data of the same experiment as in panel A but with hybridization performed with the 971-bp *Eco*RI fragment (containing the *proU* promoter region) from pAV375. (C) Equivalent to panel A except that irradiation was performed at 4°C. hns^+ (lanes 1 to 4) and *hns* (lanes 5 to 8) cells were cross-linked for 4 (lanes 1 and 5), 8 (lanes 2 and 6), 12 (lanes 3 and 7), or 16 (lanes 4 and 8) min. (D) Equivalent to panel B except that irradiation was performed at 4°C. The slopes of the relationships (i.e., rates of cross-linking) are indicated in boxes in all the graphical representations. Double-stranded (DS) and single-stranded (SS) bands are indicated.

HU, which binds DNA more tightly than H-NS, has little effect on TMP photobinding (38).

To assess whether the differences in TMP cross-linking between the *hns* and hns^+ strains were due to the presence of the *proU* promoter and DRE (with which H-NS is known to interact [27]) in the plasmid, the experiments were repeated with plasmid pFM375 (pAV375 from which the *proU* promoter and DRE are excised). The rate of cross-linking of the 4.2-kb fragment of pFM375 was always higher for the *hns* mutant than for the hns^+ strain. Moreover, the results were quantitatively equivalent to those obtained for the vector fragment of pAV375 in cells grown and treated in the same way (data not shown). Thus, the increase in supercoiling in the *hns* mutant is independent of any specific effect of H-NS on *proU* expression or interaction of H-NS with the DRE. Thus, we conclude that in vivo, *hns* mutants exhibit generally higher levels of unconstrained negative supercoiling of plasmid DNA.

The level of chromosomal DNA supercoiling is also affected in *hns* mutants. To date, the effects of H-NS on chromosomal DNA supercoiling have not been assessed. We analyzed the rate of TMP cross-linking at three regions of the *E. coli* chromosome for cells grown in LB: a 1.25-kb *Xmn*I-*Xmn*I fragment from the maltose operon, a 950-bp *Hae*III-*Hae*III fragment within the enolase gene, and a 1,045-bp *Eco*RV-*Nde*I fragment from the *proU* operon. These genes are located at 92, 60, and 57.5 min, respectively, on the chromosome, and at least the maltose operon is likely to be located in a different topological domain from the other loci (28, 39, 43).

The rate of chromosomal DNA cross-linking by TMP in vivo was determined in the logarithmic phase of growth in LB, with irradiation performed at 4°C. In the *hns*$^+$ strain, the rate of cross-linking was higher for the enolase region than for the maltose and *proU* regions, for samples from the same cell culture (1.0 compared with 0.6 [Fig. 3A and B]). Several explanations for this can be evoked. First, the *eno* gene could contain a higher number of TMP-cross-linkable sequences. However, the DNA fragments were similar in size and large enough to make this unlikely. Second, there could be differences in transcription between the genes. However, as cross-linking was performed at 4°C, transcription should not be a factor. To exclude an effect of transcription, the rates of cross-linking of the maltose fragment were compared for cultures grown LB medium and MMAA (where *mal* transcription is repressed). The rate of cross-linking was not significantly affected by repression of transcription (Fig. 3B and C). The third and most likely interpretation is that the enolase region of the chromosome has a higher basal level of supercoiling. This may reflect different levels of supercoiling of localized chromosomal domains.

For each of the three genes studied, whatever the basal level of supercoiling, the rate of cross-linking was consistently higher (1.6 to 1.7 times) in the *hns* than in the *hns*$^+$ strain (Fig. 3 and data not shown). This result implies that the *hns* mutation increases the level of unconstrained DNA supercoiling to similar extents for all regions of the chromosome. The increase in the rate of cross-linking for the maltose region in the *hns* strain was the same whether the cells were grown in LB or MMAA (where transcription is repressed). Thus, the increase in rate of cross-linking (DNA supercoiling) in the *hns* mutant cannot be a consequence of increased transcription.

Effect of *hns* mutations on TopA activity. The data shown above indicate that *hns* mutants have increased levels of negative supercoiling of both plasmid and chromosomal DNA. It is possible that H-NS influences DNA supercoiling indirectly, by decreasing topoisomerase I (TopA) activity. To exclude this possibility, we took advantage of the fact that TopA has a specific effect on the distribution of topoisomers in plasmids in which the *tetA* and *blaM* genes are divergently transcribed (3, 19, 20, 31). According to the twin-domain model (18), divergent transcription generates a local supercoiling domain which in a *topA* strain is observed as a population of oversupercoiled plasmid molecules. When the same plasmids are isolated from *topA*$^+$ strains, the oversupercoiled domain is relaxed and no bimodal distribution is observed (2, 25, 31). The population of oversupercoiled plasmids is independent of the global level of supercoiling but is entirely dependent on the activity of topoisomerase I (30, 36). Thus, if *hns* mutations influence DNA topology through inhibiting TopA activity, they would be expected to introduce this bimodal distribution, with the percentage of oversupercoiled plasmid reflecting the level of the topoisomerase activity. Plasmid pLEU500Tc was transformed into *E. coli* strains GM37 (*hns*$^+$ *topA*$^+$), GM230 (*hns topA*$^+$), and RED31 (*hns*$^+$ *topA*). Plasmid DNA was extracted from cultures in the logarithmic phase of growth, and the topoiso-

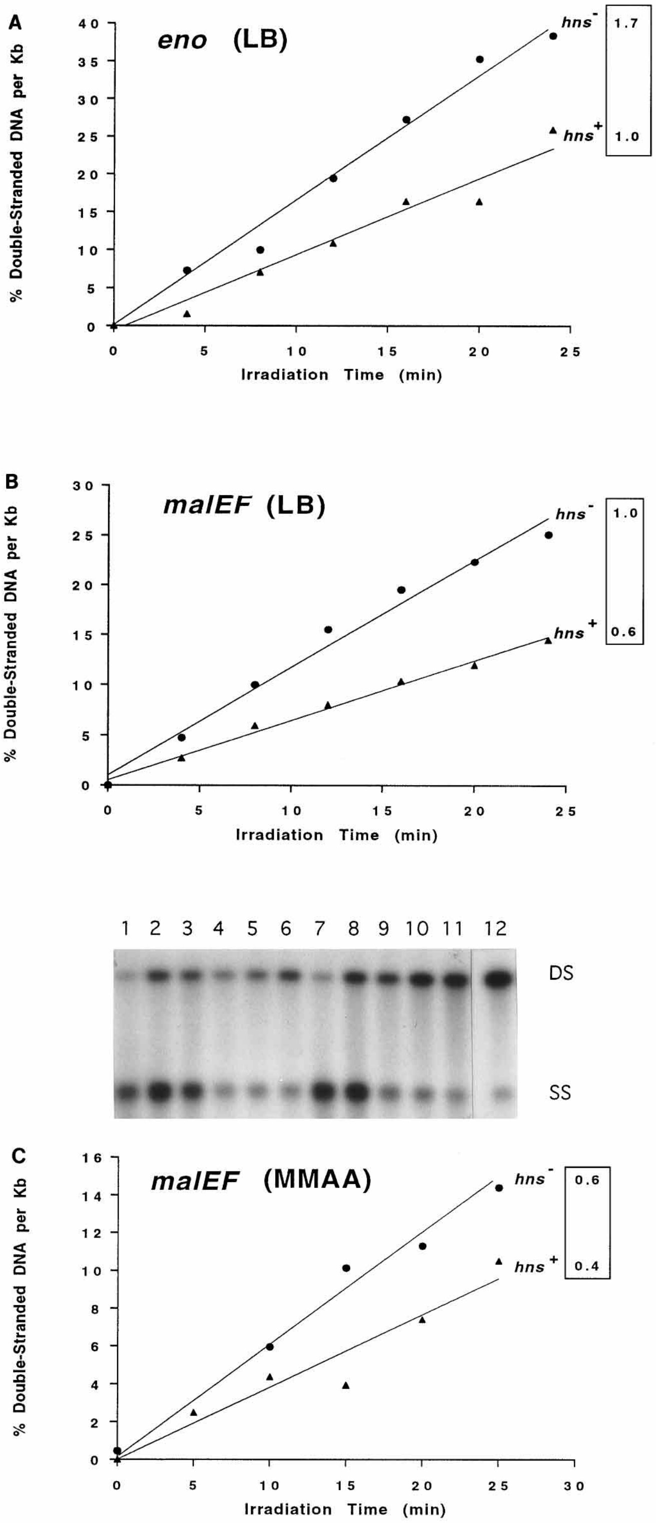

FIG. 3. TMP photo-cross-linking of chromosomal DNA from the *hns*$^+$ strain GM37 (triangles) and the *hns* mutant GM230 (circles). Irradiation was performed at 4°C. (A) Graphical representation of TMP cross-linking analysis of a 950-bp *Hae*III-*Hae*III DNA fragment within the chromosomal *eno* gene from cultures grown in LB medium. (B) Analysis of TMP cross-linking rates of a 1.25-kb *Xmn*I-*Xmn*I DNA fragment from within the *malEF* operon in the *hns*$^+$ (lanes 1 to 6) and *hns* (lanes 7–12) strains grown in LB medium. Cells were irradiated for 4 (lanes 1 and 7), 8 (lanes 2 and 8), 12 (lanes 3 and 9), 16 (lanes 4 and 10), 20 (lanes 5 and 11), or 24 (lanes 6 and 12) min. The raw data and graphical representation are shown. DS, double-stranded DNA; SS, single-stranded DNA. (C) Representation of data from an experiment equivalent to that in panel B but with cells grown in MMAA medium. The slopes of the relationships are indicated.

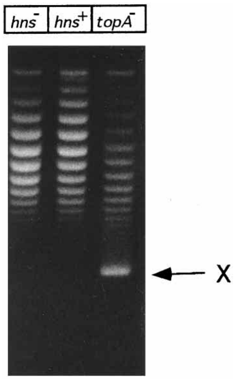

FIG. 4. Topoisomer distributions of plasmid pLEU500Tc separated by chloroquine-agarose gel electrophoresis. Plasmid DNA was isolated from logarithmic cells of the *hns topA*$^+$ (*hns*$^-$), *hns*$^+$ *topA*$^+$ (*hns*$^+$), and *hns*$^+$ *topA* (*topA*$^-$) strains GM230, GM37, and RED31, respectively, grown in LB medium. Under the conditions of the electrophoresis, more supercoiled topoisomers run faster. X indicates the oversupercoiled population of plasmids in the *topA* strain.

mer distribution was analyzed by chloroquine-agarose gel electrophoresis (Fig. 4). No oversupercoiled plasmid was detected for the *hns* mutant, although, as expected, a considerable proportion of oversupercoiled plasmid DNA was found in the *topA* strain (RED31). Thus, the *hns* mutation does not significantly affect the activity of topoisomerase I; consequently, the effects of this *hns* mutation on DNA supercoiling must be due to a mechanism other than altered topoisomerase I activity.

In conclusion, the data presented here provide direct in vivo evidence for an increase in unconstrained negative DNA supercoiling in an *hns* mutant. These data extend studies which showed that H-NS can influence plasmid DNA topology in vitro (42). They also show, for the first time, that *hns* mutations affect the topology of chromosomal DNA. These effects of H-NS on DNA topology are independent of transcription and are not mediated through effects on TopA activity. This is consistent with the fact that H-NS itself can constrain DNA supercoils (42). Precisely how the effects of H-NS on DNA topology influence transcription and chromosomal organization remains to be determined.

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