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Chromosomes in living *Escherichia coli* cells are segregated into domains of supercoiling

(DNA supercoils/DNA gyrase/coumermycin/psoralen/nascent RNA)

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ABSTRACT **Torsional tension in the DNA double helix can be detected in living cells of *Escherichia coli* from measurements of the rate of trimethylpsoralen photobinding to the intracellular DNA. Here we show that this tension is relaxed *in vivo* when single-strand DNA breaks are introduced by γ-irradiation and that approximately 160 nicks per genome equivalent of DNA are required to relax >95% of the tension. Chromosomes containing less than 160 nicks per genome equivalent lose only a part of the tension, depending on the number of nicks. The remaining tension is maintained during incubations of cells at 0°C. Chromosomes with tension relaxed by incubation of cells with inhibitors of DNA gyrase interact with the trimethylpsoralen probe independently of the number of nicks introduced by γ-irradiation. The results fit a model in which the chromosome in growing *E. coli* cells (mean generation time, 30 min) is segregated into 43 ± 10 domains of supercoiling per genome equivalent of DNA or 120 ± 30 domains per nucleoid. The number of domains is unchanged in cells depleted of nascent RNA by growth with rifampicin, but varies somewhat in cells growing at different rates in different media.**

The packaged DNA in isolated bacterial nucleoids (1) is negatively supercoiled (2) with a density of about 1 supercoil per 200 base pairs, and the DNA in this isolated chromosome is segregated into separate domains of supercoiling (2, 3). A domain of supercoiling is defined as a segment of chromosomal DNA bounded by topological constraints on the rotation of the double helix (3). The first rough estimates of the number of domains per *Escherichia coli* chromosome were calculated from the number of nicks (introduced by DNase) required to relax the supercoiling (2, 3). These studies indicated that the isolated nucleoids had 12 to 80 domains per genome equivalent of *E. coli* DNA. Later studies using more accurate methods showed that there were about 100 domains per genome equivalent (4, 5), but that chromosomes isolated from exponentially growing cells (mean generation time, 30 min) contained more than one genome equivalent of DNA (6, 7) so that there were, on average, about 280 domains per chromosome.

By use of analogous procedures, nuclear structures containing condensed DNA were later isolated from *Drosophila melanogaster* (8), mammalian (9, 10), and yeast (11) cells, and it was found that this packaged DNA was also segregated into multiple domains of supercoiling. The possible significance of the domain substructure of chromosomes and chromatin was immediately appreciated. Separate domains permit the maintenance of different degrees of DNA torsional tension in different parts of the same chromosome. Thus, it is possible to relax the torsional tension in parts of a chromosome without affecting other parts and also possible, in principle, to regulate the torsional strain independently in different domains of the same chromosome. The last consideration is especially important because there are suggestions that the DNA torsional tension strongly influences rates of DNA transcription (12–15), recombination (16–18), replication (14, 19–21), viral encapsidation (22), transposition (23), and changes in chromosome condensation (22). Thus, there could be a structural basis in chromosomes and chromatin permitting regulation of these DNA-dependent processes in different domains. Indeed, there is evidence in eukaryotes that initiation of DNA replication is regulated in units comprising many tandem replicons (24–26) and it has been proposed that the units may be equivalent to a domain (27, 28).

Evidence supporting the domain structure of chromosomes has come exclusively from studies of isolated chromosomes or nuclear structures. The stability of the isolated bacterial chromosomes and some of the eukarytotic structures is dependent on nascent RNA molecules and proteins bound to the isolated chromosomes (1–3, 8). When these chromosomes are incubated with RNase or when isolation is attempted from cells treated with inhibitors of RNA synthesis, the DNA unfolds (1–3, 8, 29) and the constraints that define domains are lost (3, 30). Investigations of the RNA components of isolated nucleoids have not revealed a unique class of chromosome-stabilizing RNA; it appears that the DNA-bound RNA molecules that interact most strongly are comprised predominantly (if not exclusively) of nascent mRNA and rRNA species (31). Thus, it has often been considered that some of the stabilizing interactions in these isolated chromosomes may not reflect the natural interactions *in vivo* (4, 5, 31–34). This raises the possibility that the observed segregation of DNA in chromosomal domains may not exist in the natural state.

To resolve this uncertainty, methods are required to investigate the domain structure of chromosomes inside living cells. Our approach to this problem has exploited trimethylpsoralen as a probe of the distribution of domains *in vivo*. Recently, we showed that this probe photobinds to duplex DNA at a rate directly proportional to the torsional tension in the double helix and that tension in the winding of the helix can be detected *in vivo* from measurements of the photobinding in living *E. coli* cells (35). Tension was relaxed *in vivo* when single-strand DNA breaks were introduced in the intracellular *E. coli* chromosome by γ-irradiation. Thus, it should be possible to quantitate the number of breaks or swivels required *in vivo* to relax all detectable torsional tension in the chromosomal DNA and to determine thereby if the chromosome in its natural state is segregated into domains. Here we show that the chromosome in living *E. coli* cells is segregated into domains and describe how the number of domains varies in cells growing in different conditions and in cells lacking nascent RNA molecules.

MATERIALS AND METHODS

Bacterial Strain and Growth Conditions. AS19, a prototropic derivative of *E. coli* B selected for permeability to actinomycin (36) which is also more permeable to trimethylpsoralen than

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most other *E. coli* strains (37), was used in these studies. Cultures were grown in K, Luria, or succinate medium as described in the text. M9 buffer and K medium were as described (35). Succinate medium (SM9) was identical to K medium except that Na_2 succinate was substituted for glucose. Luria broth contained, per of liter deionized water, 10.0 g of tryptone, 5.0 g of yeast extract, and 10.0 g of NaCl. All cultures were in exponential growth at 37°C and at a density of 2–4 × 10^8 cells per ml when cells were harvested.

γ-Irradiation of Cells. Growing cells were quickly chilled to 4°C, sedimented, resuspended in cold M9 buffer, and exposed to various doses of ^{60}Co irradiation in a Gamma Cell 220 (Atomic Energy Canada, Ottawa). Cells were held in open glass vials during irradiation. The dose rate was 5.1 krad/min (1 rad = 1.0 × 10^{-2} joules/kg). There were 8.5 single-strand DNA breaks introduced per krad per 2.7 × 10^9 daltons of DNA. This value is slightly higher than reported previously (5); however, in the present studies the bacterial strain, ^{60}Co source, and irradiation conditions were different.

Measurement of Single-Strand DNA Breaks. Spheroplasts of γ-irradiated cells, made as described (35), were layered on alkaline sucrose gradients and sedimented in the presence of intact ^{14}C-labeled T4 and T7 DNA markers. Molecular weights of the broken single-stranded *E. coli* DNA were determined and used to calculate the number of single-strand breaks per genome equivalent of DNA as described (5). This value was corrected for the 20% of the breaks that are induced by alkali (5, 38). In this analysis, double-strand breaks that occurred at a frequency 0.077 that of single-strand breaks were scored as two single-strand breaks.

Photobinding of Trimethylpsoralen. Details of the procedure for measuring amounts of photobound trimethylpsoralen have been described (35). Briefly, cells suspended in M9 buffer at 4°C were mixed with 4,5′,8-[^{3}H]trimethylpsoralen (1.2 μg/ml; 1.16 × 10^6 cpm/μg) and exposed to doses of 360-nm light of 2 and 4 kJ/m^2. Cells were converted to spheroplasts by treatment with lysozyme and EDTA and lysed by the addition of 0.5% $NaDodSO_4$. Cell lysates were extracted twice with phenol and four times with $CHCl_3$/isoamyl alcohol, 24:1 (vol/vol), and the nucleic acids were precipitated by the addition of 2 vol of ethanol. Precipitates were redissolved in 0.3 ml of 10 mM Tris, pH 7.6/1 mM EDTA/50 mM NaCl, and the RNA was digested for several hours with pancreatic RNase (30 μg/ml) and T1 RNase (15 units/ml). The digests were applied to Bio-Gel A-150 m columns (1 × 27 cm), and the DNA and digested RNA were eluted in separate bands at a flow rate of 0.25 ml/min. The specific activities of [^{3}H]trimethylpsoralen-labeled DNA and RNA were determined by measuring radioactivity and absorption at 260 nm. The ratio of the specific activities of DNA and RNA averaged over the two doses of 360 nm light is denoted *R*.

Treatment of Cells with Coumermycin. Coumermycin A1 was added to a culture of exponentially growing AS19 to a final concentration of 50 μg/ml, and incubation at 37°C was continued for 35 min. Our previous studies showed that the rate of photobinding of trimethylpsoralen to the DNA in such cells is similar to that with relaxed DNA (35).

Chemicals. 4,5′,8-Trimethylpsoralen was obtained from Paul B. Elder Co. (Bryan, OH). The [^{3}H]trimethylpsoralen was prepared commercially by catalytic exchange (New England Nuclear). Coumermycin A1 was a gift from Bristol Laboratories (Syracuse, NY). Rifampicin SV grade B was purchased from Calbiochem.

RESULTS

Detection of Negative Superhelical Tension *in Vivo*. We have shown previously that the rate of photobinding of trimethylpsoralen to closed duplex DNA is a linear function of the negative superhelical density of DNA and have applied this approach to assay the unrestrained torsional tension in chromosomal DNA *in vivo* (35). The "rate of photobinding" is expressed as the number of trimethylpsoralen adducts formed per unit time per unit mass of nucleic acid per unit dose of 360-nm light. The most accurate and reproducible analysis is in terms of a ratio, *R*, which expresses the rate of photobinding of trimethylpsoralen to DNA normalized to the rate of binding to total cellular RNA in the same cells. Analysis in this form normalizes differences in the intracellular concentration of trimethylpsoralen available in different samples for photobinding and also corrects internally for variation in doses of 360-nm light during photobinding. Because the amounts of trimethylpsoralen photobound to either the chromosomal DNA or RNA are essentially linear functions of the dose of 360-nm light (35), *R* is closely approximated by the ratio of specific activities of [^{3}H]trimethylpsoralen-labeled DNA and RNA isolated after exposure of cells to [^{3}H]-trimethylpsoralen and light.

E. coli cells that had previously been irradiated with different doses of γ-irradiation to introduce various numbers of single-strand breaks were treated with constant amounts of [^{3}H]trimethylpsoralen and light. *R* for the nucleic acids isolated from these cells depended on the number of breaks in the chromosomal DNA (Fig. 1). The decrease in *R* with increasing num-

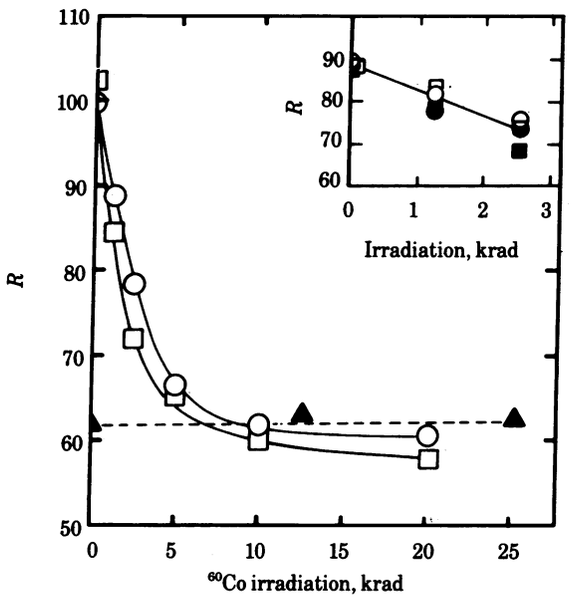

FIG. 1. Relaxation of DNA superhelical tension by γ-irradiation in living *E. coli* cells. AS19 cells in exponential growth were quickly chilled to 0°C, sedimented, resuspended in cold M9 buffer, and exposed to ^{60}Co irradiation. Samples removed after various doses of γ-irradiation were treated with [^{3}H]trimethylpsoralen and 360-nm light at incident doses of 2 and 4 kJ/m^2. Nucleic acids were purified and the specific activities of the [^{3}H]trimethylpsoralen-labeled DNA and RNA were determined. *R* is the ratio of DNA and RNA specific activities averaged over the two doses of 360-nm light. ○, Control cells; □, cells from half the control culture grown 30 min at 37°C with rifampicin (100 μg/ml). In a separate experiment, cells were incubated for 35 min in the presence of coumermycin A1 (50 μg/ml) before γ-irradiation (▲). (*Inset*) Effects of relatively low doses of γ-rays in a similar experiment. Cells were γ-irradiated and treated with trimethylpsoralen and light immediately after γ-irradiation or held for 15 min at 0°C after γ-irradiation before photobinding of trimethylpsoralen. ○, Control cells photobound immediately after γ-irradiation; □, rifampicin-treated cells and photobinding immediately after γ-irradiation; ●, control cells held 15 min after γ-irradiation before photobinding; ■, rifampicin-treated cells held 15 min before photobinding.

ber of nicks is due primarily to reduced photobinding to the DNA because changes in the photobinding to RNA are insignificant (35). Also, because the cells were held at 0°C prior to and during photobinding, there is no change in the cellular RNA/DNA mass ratios due to γ-irradiation. A minimum was reached after which introduction of additional single-strand breaks in the intracellular DNA had no further effect on the rate of psoralen photobinding. The rate of photobinding to DNA in cells incubated with coumermycin was not affected by γ-irradiation. It is known that coumermycin causes a relaxation of DNA torsional tension *in vivo* (35, 39) without introducing permanent single-strand breaks in the chromosomal DNA (39).

Thus, it seems that the introduction of single-strand breaks in chromosomal DNA by γ-irradiation has no effect on the photobinding reaction unless the DNA is initially wound with torsional tension. The results reinforce previous conclusions (35) that the DNA in living *E. coli* cells is normally wound with unrestrained torsional tension which can be relieved when swivels are introduced into the DNA by γ-irradiation.

Number of Domains of Supercoiling *in Vivo*. Torsional tension determined from the rate of trimethylpsoralen photobinding was lost progressively as the number of single-strand breaks introduced into the chromosome increased (Figs. 1 and 2). Because multiple nicks were required to complete the relaxation, it appeared that independent domains of DNA supercoiling exist *in vivo*. The *R* value resulting from a given number of single-strand breaks did not vary when cells containing partially relaxed chromosomes were held at 0°C after γ-irradiation (Fig. 1 *Inset*). Thus, it seems that the partial relaxation obtained from a given number of nicks had gone to completion and could not be attributed to slow kinetics of relaxation. This is exactly as expected if the relaxation occurred in an all-or-none fashion only in domains containing a swivel and was not propagated into adjacent domains lacking a swivel.

To determine the average number of domains of supercoiling

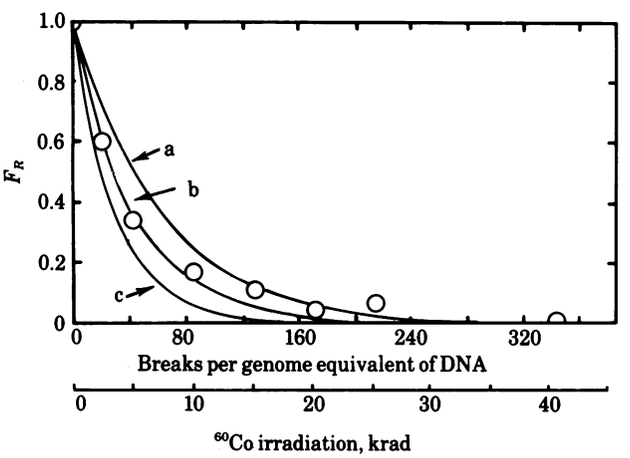

FIG. 2. Determination of number of domains of supercoiling per genome equivalent of DNA. *R* values were measured in an experiment similar to that in Fig. 1. Data are presented as F_R, the fractional change in *R* relative to the change between unirradiated cells and cells with fully relaxed chromosomal DNA (extensive γ-irradiation). Because the change in *R* represents the difference in the rate of photobinding to chromosomes containing supercoiled or nicked domains, we assume that F_R describes the fraction of intact domains remaining in the chromosome: $F_R = e^{-(x/m)}$ in which *x* is the number of nicks and *m* is the total number of domains of supercoiling per genome-sized duplex DNA molecule. The number of nicks introduced was determined by sedimentation of the DNA from γ-irradiated cells on alkaline sucrose gradients. ○, data points. Lines are theoretical relationships predicted from the above equation corresponding to 30 (curve c), 43 (curve b), and 60 (curve a) domains per genome equivalent of DNA.

(*m*) per genome equivalent of DNA *in vivo*, we assumed the simplest model, that the domains are of equal size and each is wound with the same density of unrestrained supercoils. We also assumed that the distribution of nicks introduced into the domains by γ-irradiation is described by a Poisson distribution. If a single nick per domain is sufficient to relax all tension in that domain, the fraction of the original tension remaining in the chromosome after the introduction of a defined number of nicks will be described by the zero-order term of the Poisson distribution, or the fraction of domains containing no nicks, $p(0) = e^{-(x/m)}$, in which *x* is the number of nicks introduced per genome equivalent of DNA. Support for the assumption of equal-sized domains comes from the observation that the rate of decay of torsional tension with numbers of single-strand hits is close to first-order (Figs. 1 and 2). Theoretical relationships can then be constructed which relate different values of *m* to the change in *R* with the average number of single-strand breaks per genome equivalent of DNA (Fig. 2). In the analysis shown, the curve for a value of 43 domains per genome equivalent is the best fit. The deviation of the experimental points from the theoretical curve at high numbers of nicks per genome equivalent is within the experimental variability.

Numerous repeats of this experiment have shown a reproducibility of about ±10 domains per genome equivalent. For example, the data given in Fig. 1 fit best with $m = 33$. Results averaged over all data indicate about 43 ± 10 domains per genome equivalent of DNA. Because nucleoids in cells grown in similar conditions contain 2.8 genome equivalents of DNA (7), there should be about 120 ± 30 domains per bacterial chromosome *in vivo*. This value does not differ greatly from the number of domains estimated with isolated nucleoids (4, 5).

Variation in Number of Domains per Chromosome. When repeated measurements of the number of domains were made on chromosomes in cells from the same culture, reproducibility was greater than ±10 domains (for example, see Fig. 1). It therefore seemed possible that variability in different cultures might be attributable in part to uncontrolled variations in growth conditions. To determine if the number of domains varied in cells grown in different media, cells were grown in succinate, K, or Luria broth media at 37°C. Cultures were inoculated so that all three cultures reached a density of 2–4 × 10^8 cells per ml at the same time. Cells were harvested, γ-irradiated, and treated with [^{3}H]trimethylpsoralen and light as before, and the *R* values were determined.

Data from a representative experiment are shown in Fig. 3. There was little difference between the numbers of domains in cells grown in K medium or in Luria broth, but cells grown in succinate medium consistently had fewer domains (about 25 domains) than did cells grown in richer media. The frequency of DNA nicks introduced by a given dose of γ-irradiation was essentially the same in cells grown in succinate or in K medium. The fractional decrease in *R* in repeats of this experiment was 0.7 in cells grown in succinate and 0.6 in cells grown in K medium. This could be explained if the density of unrestrained supercoils were slightly less in chromosomes of cells grown in K medium; however, additional studies will be required to confirm this interpretation. The results indicate that the interactions defining the boundaries of domains may be dynamic and may vary in cells growing at different rates.

Number of Domains in Cells Depleted of Nascent RNA. To determine if nascent RNA molecules are involved in the stabilization of the domains of supercoiling *in vivo*, exponentially growing cells were incubated with rifampicin to eliminate nascent RNA, and the number of nicks required to relax torsional tension in the chromosome was determined. If nascent RNA molecules are required to stabilize domains of supercoiling *in*

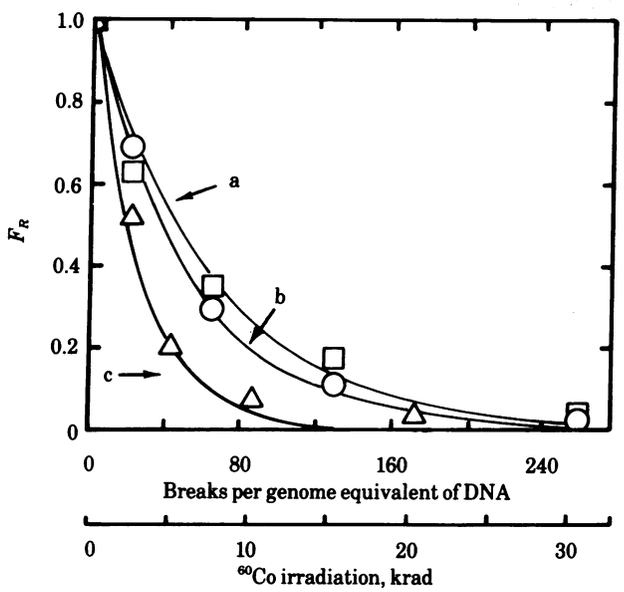

FIG. 3. Variation in the number of domains of supercoiling in cells growing in different medium. Results from an experiment similar to that of Fig. 2 are shown in which AS19 cells were grown in succinate medium (△), K medium (○), or Luria broth (□). The curves are theoretical lines corresponding to 27 (curve c), 54 (curve b), and 65 (curve a) domains of supercoiling per genome equivalent of DNA.

vivo, fewer nicks should be required to relax the DNA tension after treatment with rifampicin. There was no difference in the rate of photobinding to chromosomal DNA in control and rifampicin-treated cells (Fig. 1). This suggests that the domains of supercoiling in chromosomes of living cells are not stabilized by nascent RNA molecules. Control experiments showed that the rate of [^{3}H]uridine incorporation was inhibited more than 92% by 30 min of rifampicin treatment. Also, sedimentation studies on alkaline sucrose gradients showed that DNA from rifampicin-treated or control cells γ-irradiated with the same dose had the same number of nicks.

To determine the *in vivo* stability of partially relaxed chromosomes in control and rifampicin-treated cells, samples were treated with low doses of γ-irradiation and held for different times before treatment with trimethylpsoralen and light (Fig. 1 *Inset*). There was no detectable difference in the photobinding to partially relaxed chromosomes in control or rifampicin-treated cells when photobinding was delayed. Thus, the partially relaxed chromosomes, containing a mixture of nicked and supercoiled domains, are stable in cells for at least 15 min after the γ-irradiation, and the stability is not dependent on nascent RNA molecules.

DISCUSSION

Data presented here show that DNA in the *E. coli* chromosome is organized *in vivo* into independent supercoiled domains. Thus, different levels of torsional tension in the winding of the double helix can be maintained in different parts of the same chromosome in a living cell. A swivel introduced into a specific site in the chromosome during DNA recombination, repair, or replication, for example, will relax the torsional tension in only one domain without affecting the bulk of the chromosome. Whether or not different states of torsional strain are actually maintained in separate domains is unknown, but the present results indicate that regulation at this level is feasible structurally.

The number of domains per genome equivalent seemed to vary somewhat in cells growing in different media. The variation was in the opposite direction expected if there were a constant number of constraints defining domains per cell. For example, cells growing in succinate medium (mean generation time, 60 min) had about 20 fewer domains per genome equivalent and even fewer domains per chromosome than cells growing in Luria broth (mean generation time, 25 min) which have, on average, more DNA per cell and more DNA per chromosome. It seems, therefore, that the absolute number of interactions defining the limits of domains vary in cells growing at different rates. The actual number of domains per genome equivalent in cells growing in K medium was 43 ± 10. This number agrees, within a factor of 2, with the number estimated from studies of nucleoids isolated from cells growing at similar rates (5). Thus, even though the interactions defining the domains may be rearranged during isolation of chromosomes, the structure seems to be preserved with respect to this quantitative feature.

Several models for the structure of the bacterial chromosome have been proposed based on studies of isolated nucleoids. Because the present results indicate that the constraints defining domains are independent of nascent RNA molecules, they do not support models proposing stabilization by such RNA (3). The stabilizing RNA–DNA interactions that occur *in vitro* in the isolated chromosome may occur as a result of R looping or other aggregations induced during isolation, as previously considered (4, 31, 32). Nevertheless, these interactions do stabilize the chromosome in a structure having similar size, DNA content, and number of domains as the chromosome in its natural state (32), although they may lead to an inside-out inversion of the nucleoid DNA (4). Our data do not rule out the possibility that stable, non-nascent RNA molecules are involved in chromosome structure *in vivo*, although an extensive search failed to find such molecules in isolated nucleoids (31).

The results are also compatible with models in which domains are defined by direct DNA–membrane interactions (40) or are stabilized by proteins such as DNA gyrase complexes which could link separate DNA segments of the same chromosome (39, 41, 42). Models which proposed linkage of the chromosome to the cell envelope via the complex of nascent mRNA, ribosomes, and nascent peptide chain (29, 34, 40) may have to be reconsidered. Although our results do not argue against the existence of such complexes, they suggest that they are not critical in defining domains.

The procedures using trimethylpsoralen to probe the domain structure of chromosomes may also be applicable to eukaryotic systems. Net torsional tension in intracellular DNA has not yet been detected in the eukaryotic cells that have been studied (35). However, suitable modifications of this approach may open the way for investigations of the domain organization in chromosomes lacking natural torsional tension.

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