JOURNAL OF BACTERIOLOGY, Feb. 1995, p. 1094–1097
0021-9193/95/$04.00+0
Copyright © 1995, American Society for Microbiology
Vol. 177, No. 4

# Thermoregulation of *virB* Transcription in *Shigella flexneri* by Sensing of Changes in Local DNA Superhelicity

TORU TOBE,* MASANOSUKE YOSHIKAWA, AND CHIHIRO SASAKAWA

*Department of Bacteriology, Institute of Medical Science, University of Tokyo, Minato-ku, Tokyo 108, Japan*

Received 6 September 1994/Accepted 26 November 1994

**Transcription of the *virB* gene, a transcriptional regulator of invasion genes on the large plasmid of *Shigella flexneri*, is strictly regulated by growth temperature; when bacteria are grown at 37°C, *virB* transcription is highly activated, while at 30°C the level of *virB* transcription decreases to less than 5% of that at 37°C. Transcription from the *virB* promoter is activated by VirF, which is encoded on the same plasmid, in a DNA superhelicity-dependent manner (T. Tobe, M. Yoshikawa, T. Mizuno, and C. Sasakawa, J. Bacteriol. 175:6142–6149, 1993). Here we provide evidence supporting the involvement of negative superhelicity in the thermoregulation of *virB* transcription. A local negatively supercoiled domain in the *virB* promoter region was created by activating a divergent transcription from the T7 RNA polymerase-dependent promoter, φ10, which was placed upstream of the *virB* promoter in the opposite orientation. Transcription from the *virB* promoter was activated even at 30°C by induction of divergent transcription. Levels of *virB* transcription correlated with levels of expressed T7 RNA polymerase. Transcriptional activation of *virB* by the system depended completely upon VirF function. The level of *virB* transcription achieved by introducing a negatively supercoiled domain was enough to give rise to expression of invasion capacity at 30°C. These results indicated that the repression of *virB* transcription at 30°C was caused by a reduction in negative superhelicity around the *virB* promoter region at 30°C.**

Temperature is a well-known signal for virulence gene expression in a wide variety of pathogens. Expression of *Shigella* virulence has been shown to be dependent upon growth temperature (8). This thermoregulated expression is due mainly to temperature-dependent transcription of a regulatory gene, *virB*, the positive regulator for transcription of invasion operons, *ipa*, *mxi* (Region 3, 4) and *spa* (Region 5) (13). The transcription of *virB* at 30°C is less than 1/20 of that at 37°C (13). Transcription from the *virB* promoter depends on another regulator, VirF, which is encoded by the *virF* gene on the same large plasmid (1), although transcriptional activation by VirF at 30°C is inefficient compared with that at 37°C (13). Recent studies have indicated that *virB* transcription is activated by binding of the VirF protein to a sequence upstream of the *virB* promoter and that this transcriptional activation by VirF is highly responsive to changes in DNA superhelicity (14). Indeed, DNA superhelicity in *Shigella flexneri* has been shown to be affected by temperature (5), in that reporter plasmids have been shown to be in a more negatively supercoiled state at 37°C than at 30°C (15). On the other hand, derepression of *virB* transcription at 30°C has been observed in the *hns* mutant (14). The *hns* mutation has been shown to affect DNA superhelicity (5), while it has also been suggested that H-NS protein inhibits *virB* transcription by binding to the *virB* promoter directly (14). To demonstrate the role of DNA superhelicity at the *virB* promoter region alone in thermoregulated *virB* transcription, we constructed a system to introduce a negatively supercoiled domain into the *virB* promoter region in vivo and examined the effect on *virB* transcription.

**Construction of a system to introduce a local negatively supercoiled domain.** It has been indicated that translocation of an RNA polymerase elongation complex with a large molecule along a DNA template generates two distinct supercoiled domains, one behind and one in front of the complex; the front region is more positively supercoiled than the nontranscribed template, while behind the complex is a more negatively supercoiled domain (Fig. 1A) (7, 16). Since changes in DNA superhelicity created by transcription are quickly canceled in vivo through the action of topoisomerases, these changes would be expected to occur in a very limited region around the transcription complex (3). By using this effect, local DNA superhelicity around the *virB* promoter region was changed by activating transcription from a controllable promoter placed upstream of *virB* promoter in the opposite direction, leading to the creation of a negatively supercoiled domain (Fig. 1A).

A controllable gene was constructed by deleting an *Ssp*I-*Eco*RI fragment containing the promoter of the *bla* gene from pT7-3 (12). The *Pvu*II-*Hae*II fragment containing a phage T7 φ10 promoter-*bla* fusion gene was excised from the resulting plasmid and inserted into plasmid pTB701, which is a pBR322-derived plasmid possessing the intact *virB* gene together with the upstream element essential for transcriptional activation by VirF. pTB702 was constructed by inserting the φ10-*bla* fusion gene upstream of the *virB* promoter in the opposite orientation so that the T7 φ10 promoter was at a position 154 bp away from the *virB* promoter. pTB703 and pTB704 were constructed by inserting the φ10-*bla* gene downstream of the *virB* gene in the same and opposite orientations as the *virB* gene, respectively. The T7 RNA polymerase was provided from another plasmid, pTB711, which is a derivative of pGP1-2 (12) carrying the *lacI*$^q$ gene, in which expression of T7 RNA polymerase was repressed by λ*c*I857 while the expression of λ*c*I857 was repressed by LacI. Therefore, bacteria harboring pTB711 could be made to produce T7 RNA polymerase by removing IPTG (isopropyl-β-D-thiogalactoside) from the medium. Furthermore, by using this system, transcription of the φ10-*bla* gene could be induced without adding IPTG or expressing λ*c*I857.

* Corresponding author. Mailing address: Howard Hughes Medical Institute, Beckman Center, Room B239, Stanford University Medical Center, Stanford, CA 94305-5428. Phone: (415) 723-7026. Fax: (415) 723-1399.

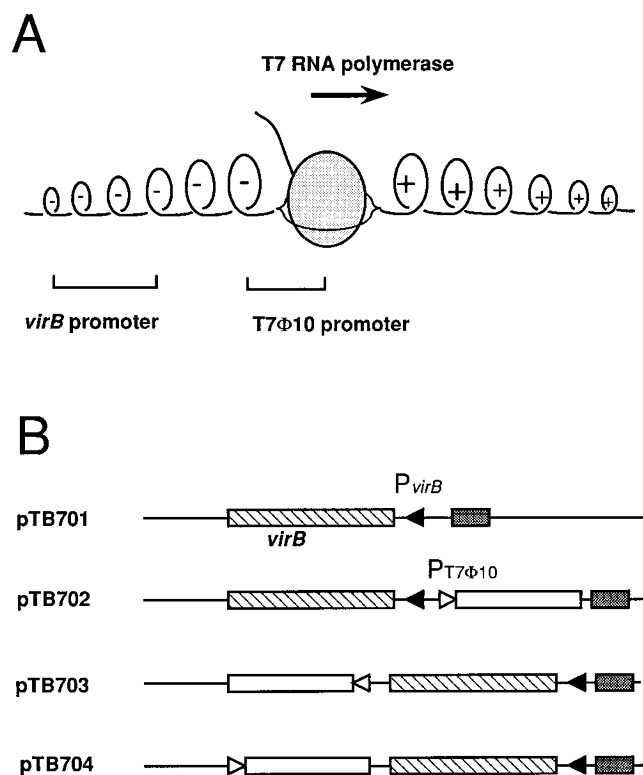


FIG. 1. Strategy for and construction of the system for introducing a local negatively supercoiled domain in the *virB* promoter region. (A) Production of a negatively supercoiled domain by divergent transcription. When transcription from the T7 phage promoter was activated, a negatively supercoiled domain was created behind the transcription elongation complex, but the effect was canceled in accordance with distance from the complex by the action of DNA topoisomerases. When the T7 promoter was placed upstream of the *virB* promoter in the opposite orientation, a negatively supercoiled domain was created in the *virB* promoter region. (B) Construction of *virB*-T7 promoter plasmids. pTB701 is a pBR322-derived plasmid which contains the *virB* gene (hatched boxes) and $rrnBt_1t_2$ transcription terminator sequence (2) (shaded box) to prevent transcription read-through from upstream. Plasmids pTB702, pTB703, and pTB704 were constructed from pTB701. pTB702 was constructed by inserting the T7 ϕ10 promoter (open triangles)-*bla* (open boxes) fusion gene upstream of the *virB* promoter (closed triangles). pTB703 has a ϕ10-*bla* fusion gene downstream of *virB* in the same orientation. pTB704 has a ϕ10-*bla* fusion gene downstream of *virB* in the opposite orientation. The distances between the transcription start sites of the *virB* and ϕ10 promoters in plasmids pTB702, pTB703, and pTB704 are 154 bp, 1.5 kbp, and 2.3 kbp, respectively.

**Activation of *virB* transcription by induction of divergent transcription.** Total RNA was extracted from bacteria grown to log phase, and *virB*-specific transcripts were detected by the primer extension method with $^{32}$P-labeled primer hybridizing with the sequence from position +57 to +76 (numbered from the transcription start site) of *virB* (14). When YSH6000T (a wild-type strain of *S. flexneri* 2a) (9) harboring pTB711 and pTB701 was grown at 30°C, *virB* transcription was greatly reduced compared with the high-level transcription at 37°C (Fig. 2, lanes 1 and 2). When transcription from the divergent T7 promoter was activated by growing bacteria in medium without IPTG, transcription of the *virB* gene on pTB702 was apparently increased even at 30°C (Fig. 2, lane 3). The transcription start site from pTB702 was the same as that from pTB701 at 37°C. Even when transcription from the T7 promoter was activated on pTB703 and pTB704, transcription from the *virB* gene was not activated on pTB703 and pTB704 (Fig. 2, lanes 4 and 5). The level of *virB* transcription from pTB702 at 30°C was one-fourth of that from pTB701 at 37°C. Since transcription of the *virF* gene at 30°C was decreased to one-fourth of that at 37°C (13), the level of *virB* transcription from pTB702 at 30°C was expected to result from the low level of *virF* at 30°C compared with that at 37°C. These results clearly indicate that transcription from the *virB* promoter was activated at 30°C by introduction of divergent transcription from the T7 ϕ10 promoter placed upstream of the *virB* promoter and that the effect was related to the position of the T7 ϕ10 promoter.

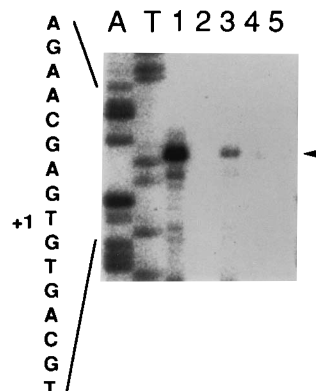


FIG. 2. Transcriptional activation from the *virB* promoter by introduction of divergent transcription. RNA was extracted from *S. flexneri* YSH6000T, which harbored plasmids pTB711 and one of pTB701 to pTB704 and which was grown in L broth (without IPTG) to log phase. *virB*-specific transcripts (arrowhead) were detected by the primer extension method with a $^{32}$P-end-labeled primer. *virB* transcription at 37°C in the strain harboring pTB701 (lane 1) and at 30°C in the strain with pTB701 (lane 2), pTB702 (lane 3), pTB703 (lane 4), or pTB704, (lane 5) is shown. A and T, sequence created with the same primer. The sequence around the transcription start site (+1) is shown on the left.

In order to clarify the relationship of the activity of the *virB* promoter to the density of negative superhelicity, various levels of divergent transcription from the T7 ϕ10 promoter were achieved by either changing the concentration of IPTG in the medium or excluding pTB711 and then examining the levels of *virB* transcription (Fig. 3A). The amount of *virB* transcription decreased in correspondence with the increase in IPTG concentration and was greatly reduced in the absence of pTB711.

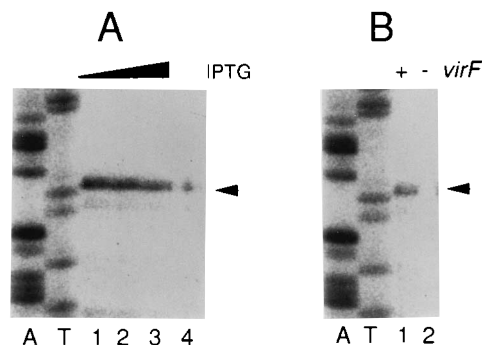


FIG. 3. Dependence of *virB* transcription on T7 promoter activity and *virF*. (A) Dosage effect of T7 RNA polymerase production on *virB* promoter activity. Bacteria harboring pTB711 and pTB702 were grown in L broth containing 0 to 1.0 mM IPTG at 30°C, and *virB*-specific transcripts were analyzed as described for Fig. 2. *virB* transcription in bacteria grown without IPTG (lane 1), with 0.05 mM (lane 2) or 1.0 mM (lane 3) IPTG, or harboring only pTB702 (without pTB711) (lane 4) is shown. (B) *virB* transcription in the presence or absence of *virF*. YSH6000T or YSH6109T harboring pTB711 and pTB702 was grown at 30°C to log phase, and *virB*-specific transcripts were detected as described for Fig. 2. Lane 1, YSH6000T (*virF*$^+$); lane 2, YSH6109T (Δ*virF*). A and T, sequence created with the same primer. Arrowhead, *virB*-specific transcript.

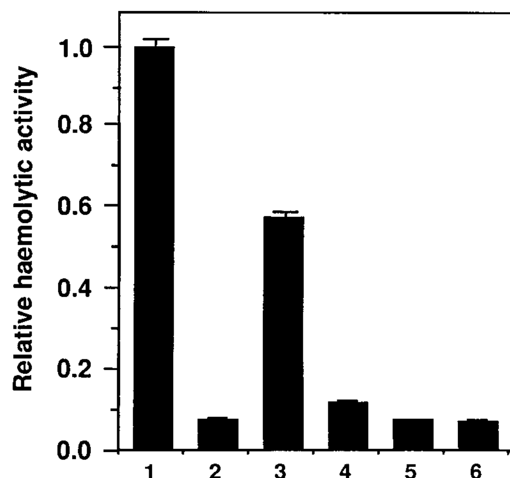


FIG. 4. Expression of invasion capacity at 30°C by activation of divergent transcription. The invasion capacity of bacteria grown at 37 or 30°C was measured by contact hemolysis assay (10). After incubation at 37°C in phosphate-buffered saline, supernatant was recovered by centrifugation and $A_{575}$ was measured. Hemolytic activity is represented as a value relative to that of YSH6000T/pTB711/pTB701 grown at 37°C. The values are averages for three independent experiments. Bars: 1, YSH6000T/pTB711/pTB701 at 37°C; 2, YSH6000T/pTB711/pTB701 at 30°C; 3, YSH6000T/pTB711/pTB702 at 30°C; 4, YSH6000T/pTB711/pTB703 at 30°C; 5, YSH6000T/pTB711/pTB704 at 30°C; 6, YSH6109T (Δ*virF*)/pTB711/pTB702 at 30°C.

This implied that the activity of the *virB* promoter was closely related to the density of DNA negative superhelicity. We next asked whether *virB* transcription required VirF function. pTB702 and pTB711 were introduced into a *virF* deletion mutant, YSH6109T (14), and *virB* transcription was examined. In the absence of the *virF* gene, transcription from the *virB* promoter was not activated at 30°C even when divergent transcription from the T7 ϕ10 promoter was activated (Fig. 3B). These results indicated that activation of transcription from the *virB* promoter at 30°C by creation of a negatively supercoiled domain was dependent upon VirF function.

**Induction of the invasion phenotype by creation of a local negatively supercoiled domain.** To examine whether the deregulated expression of the invasion phenotype could be achieved by introducing only a negatively supercoiled domain into the *virB* promoter region, YSH6000T harboring pTB711 and each of the plasmids pTB701 to pTB704 was grown at 30°C to exponential phase in the absence of IPTG, and the ability of the bacteria to invade MK2 (rhesus monkey kidney) cells (11) was tested. None of bacteria harboring pTB701, pTB703, or pTB704 were internalized by epithelial cells, whereas bacteria harboring pTB702 were capable of cell invasion (data not shown). When the invasion capacity was measured by contact hemolysis assay (10), the hemolytic activity of the strain harboring pTB702 and grown at 30°C was shown to be 58% of that of the strain harboring pTB701 and grown at 37°C (Fig. 4). Conversely, the activity at 30°C in the strains harboring pTB701, pTB703, or pTB704 was less than 10%. Since pTB702 and pTB711 were introduced into YSH6109T (Δ*virF*) and the activity was less than 10% (Fig. 4), the expression of hemolytic activity in the strain harboring pTB702 depended completely on *virF*. These results indicated that thermoregulated expression of the invasion phenotype was deregulated by the creation of a negatively supercoiled domain in the promoter region of the *virB* gene.

**Efficiency of binding of VirF and H-NS to their binding sites in the *virB* promoter at 37 or 30°C.** To examine the effect of temperature on the efficiency of binding of VirF or H-NS to its binding site in the *virB* promoter region, we constructed an assay system to measure the relative binding efficiency for each binding site. This system was based on the transcription-interfering effect of protein bound near the transcription start site (6). The *lac* promoter without the LacI repressor binding site, which consisted of the sequence from position −123 to +4 relative to the transcriptional start site (a *Pvu*II-*Eco*RI fragment created by PCR), was fused with a promoterless *trp'*-*'lacZ* gene (*Eco*RI-*Not*I fragment from pUJ8 [4]) cloned on pBluescript SKII+ (Stratagene). To prevent read-through into the fusion gene, the transcription terminator from the *rrnB* operon of *Escherichia coli* (2) was placed upstream of the promoter, and the resulting plasmid was designated pTB720. A binding site in the *virB* promoter region for VirF (positions −106 to −31 [14]) and a binding site for H-NS (−33 to +19 [14]) were inserted into the *Eco*RI site of pTB720, and the resulting plasmids, pTB720F and pTB720H, respectively, were introduced into YSH6200T (Δ*virF*) (14), YSH6200T harboring cloned *virF* (*virF*$^+$), YSH6200T (*hns*$^+$), and YSH6200T Δ*hns*::Km$^r$ (14). The β-galactosidase activity expressed in each strain grown at 37 or 30°C was then assayed. Inhibition of *trp'*-*'lacZ* transcription by VirF binding was observed at both 37 and 30°C in the *virF*$^+$ strain, and the efficiencies were essentially the same at both temperatures (30% at 37°C and 35% at 30°C). Inhibition by H-NS binding was also observed at both temperatures in the *hns*$^+$ strain, and the efficiencies were the same at both temperatures (23% at 37°C and 26% at 30°C). These results suggested that the binding of VirF or H-NS to its binding site in the *virB* promoter region was not substantially affected by the two different temperatures.

**Concluding remarks.** The introduction of a negatively supercoiled domain by divergent transcription resulted in activation of transcription from the *virB* promoter at 30°C. Since transcription directed by T7 RNA polymerase is specific for the T7 ϕ10 promoter, this system was not expected to affect promoters other than the T7 ϕ10 promoter and hence the expression of any other genes, including *virF* and *hns*. Therefore, the above-mentioned result indicated that the change of local DNA superhelicity to a more negatively supercoiled state alone is enough for activation of *virB* transcription. The wild-type *virB* gene is located downstream of *ipa* operons, and no promoter whose activity is affected by temperature has been identified upstream of the *virB* promoter on the large plasmid, indicating that changes in DNA superhelicity in the wild-type *virB* promoter region caused by temperature should be derived from total changes in the density of negative superhelicity. The activation of the *virB* promoter by this system at 30°C was one-fourth of that at 37°C and was not observed in the absence of *virF*, indicating that VirF protein molecules expressed at 30°C are in as active a state as those expressed at 37°C. Hence, repression of transcription from the *virB* promoter at 30°C is likely to result from a change of DNA superhelicity to a more relaxed state which is less preferable for transcriptional activation by VirF.

The results of the binding assay for VirF or H-NS suggested that binding of either protein to its binding site was not affected by temperature. Since binding of the H-NS protein to the *virB* promoter has been shown to inhibit transcription initiation directly (14), the inhibitory effect of H-NS on the *virB* promoter would not be different at 37 and 30°C. Furthermore, the H-NS binding site in the *virB* promoter region does not overlap with the VirF binding site (14), suggesting that binding of the VirF protein upstream of the *virB* promoter is not inhibited by binding of the H-NS protein. The most likely mechanism is that interaction of the VirF protein with RNA

polymerase is weak on relatively relaxed DNA at 30°C, and hence, transcriptional activation does not occur efficiently. Thus, we propose the following model of the mechanism for thermoregulation of the *virB* promoter. The transcriptional activation by VirF is weaker at 30 than at 37°C because of lower negative superhelicity around the *virB* promoter region at 30°C; at 30°C it is too weak to overcome the inhibitory effect of H-NS protein on transcription initiation. Consequently, transcription from the *virB* promoter is repressed to a very low level at 30°C.

We are grateful to Ruairi Mac Siomoin for critical reading of the manuscript.

This study was supported by grants 06454204, 04454194, and 06770197 from the Ministry of Education, Science and Culture of the Japanese Government.

## REFERENCES

1. **Adler, B., C. Sasakawa, T. Tobe, S. Makino, K. Komatsu, and M. Yoshikawa.** 1989. A dual transcriptional activation system for the 230 kb plasmid genes coding for virulence-associated antigens of *Shigella flexneri*. Mol. Microbiol. **3:**627–635.
2. **Brosius, J., T. J. Dull, D. D. Sleeter, and H. F. Noller.** 1981. Gene organization and primary structure of a ribosomal RNA operon from *Escherichia coli*. J. Mol. Biol. **148:**107–127.
3. **Cook, D. N., D. Ma, N. G. Pon, and J. E. Hearst.** 1992. Dynamics of DNA supercoiling by transcription in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **89:**10603–10607.
4. **de Lorenzo, V., M. Herrero, U. Jakubzik, and K. N. Timmis.** 1990. Mini-Tn*5* transposon derivatives for insertion of cloned DNA in gram-negative eubacteria. J. Bacteriol. **172:**6568–6572.
5. **Dorman, C. J., N. N. Bhriain, and C. F. Higgins.** 1990. DNA supercoiling and environmental regulation of virulence gene expression in *Shigella flexneri*. Nature (London) **344:**789–792.
6. **Elledge, S. J., and R. W. Davis.** 1989. Position and density effects on repression by stationary and mobile DNA-binding proteins. Genes Dev. **3:**185–197.
7. **Liu, L. F., and J. C. Wang.** 1987. Supercoiling of the DNA template during transcription. Proc. Natl. Acad. Sci. USA **84:**7024–7027.
8. **Maurelli, A. T., B. Blackmon, and R. Curtiss III.** 1984. Temperature-dependent expression of virulence genes in *Shigella* species. Infect. Immun. **43:** 195–201.
9. **Nakata, N., C. Sasakawa, N. Okada, T. Tobe, I. Fukuda, T. Suzuki, K. Komatsu, and M. Yoshikawa.** 1992. Identification and characterization of *virK*, a virulence-associated large plasmid gene essential for intercellular spreading of *Shigella flexneri*. Mol. Microbiol. **6:**2387–2395.
10. **Sansonetti, P. J., A. Ryter, P. Clerc, A. T. Maurelli, and J. Mounier.** 1986. Multiplication of *Shigella flexneri* within HeLa cells: lysis of the phagocytic vacuole and plasmid-mediated contact hemolysis. Infect. Immun. **51:**461–469.
11. **Sasakawa, C., K. Kamata, T. Sakai, S. Y. Murayama, S. Makino, and M. Yoshikawa.** 1986. Molecular alteration of the 140-megadalton plasmid associated with the loss of virulence and Congo red binding activity in *Shigella flexneri*. Infect. Immun. **51:**470–475.
12. **Tabor, S., and C. C. Richardson.** 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. Proc. Natl. Acad. Sci. USA **82:**1074–1078.
13. **Tobe, T., S. Nagai, N. Okada, B. Adler, M. Yoshikawa, and C. Sasakawa.** 1991. Temperature-regulated expression of invasion genes in *Shigella flexneri* is controlled through the transcriptional activation of the *virB* gene on the large plasmid. Mol. Microbiol. **5:**887–893.
14. **Tobe, T., M. Yoshikawa, T. Mizuno, and C. Sasakawa.** 1993. Transcriptional control of the invasion regulatory gene *virB* of *Shigella flexneri*: activation by VirF and repression by H-NS. J. Bacteriol **175:**6142–6149.
15. **Tobe, T., M. Yoshikawa, and C. Sasakawa.** 1994. Deregulation of temperature-dependent transcription of the invasion regulatory gene, *virB*, in *Shigella* by *rho* mutation. Mol. Microbiol. **12:**267–276.
16. **Wu, H.-Y., S. Shyy, J. C. Wang, and L. F. Liu.** 1988. Transcription generates positive and negative supercoiled domains in the template. Cell **53:**433–440.