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Binding of the transcription activator NR_I (NTRC) to a supercoiled DNA segment imitates association with the natural enhancer: An electron microscopic investigation

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ABSTRACT **Electron microscopic visualization indicates that the transcription activator NR_I (NTRC) binds with exceptional selectivity and efficiency to a sequence-induced superhelical (spiral) segment inserted upstream of the *glnA* promoter, accounting for its observed ability to substitute for the natural *glnA* enhancer. The cooperative binding of NR_I to the spiral insert leads to protein oligomerization which, at higher concentration, promotes selective coating of the entire superhelical segment with protein. Localization of NR_I at apical loops is observed with negatively supercoiled plasmid DNA. With a linear plasmid, bending of DNA is observed. We confirm that NR_I is a DNA-bending protein, consistent with its high affinity for spiral DNA. These results prove that spiral DNA without any homology to the NR_I-binding sequence site can substitute for the *glnA* enhancer by promoting cooperative activator binding to DNA and facilitating protein oligomerization. Similar mechanisms might apply to other prokaryotic and eukaryotic activator proteins that share the ability to bend DNA and act efficiently as multimers.**

Transcriptional enhancers were first noted in eukaryotes (1, 2) but also have been found in bacteria (3) and in phages (24). Enhancers have important and diverse roles in transcriptional control (4, 5). The best-studied prokaryotic system consists of σ^{54}–holoenzyme and the enhancer-binding protein NR_I (NTRC) of enteric bacteria; NR_I binds to the enhancer located upstream of a promoter such as *glnA*p2 and activates transcription by stimulating the isomerization of the closed σ^{54}–holoenzyme–promoter complex to the open state in the presence of ATP (6–8). It was shown previously that the insertion of a segment with sequence-induced superhelicity (refs. 9–10; B.R., E. Niedochodowiez, S.B., and G.B., unpublished work) upstream of the *glnA*p2 promoter substitutes for the enhancer in activating transcription with NR_I and σ^{54}–holoenzyme (11). The electron microscopy (EM) analysis presented here was made to discern the mechanism that enables a plectonemic spiral DNA insert to replace the enhancer in stimulating transcription.

MATERIALS AND METHODS

Plasmids. The plasmid pSB10 (3741 bp) contains a $(CA_5TGCC)_{56}$ repeat inserted 48 bp upstream from the *glnA*p2 promoter and is devoid of all natural NR_I-binding sites (11). The plasmid pSB21E carries a spiral $(A_6TATATA_5TCTCT)_{59}$ repeat without any sequence homology to the NR_I strong binding site sequence (enhancer) (see ref. 12). For the investigation of superhelical chirality, plasmids were constructed to contain inserts with sequence repeats of 10.0, 10.5, and 11.0 bp. The inserted fragments are multimers of the type A_5N_5, $A_6N_5A_5N_5$, and A_6N_5, respectively (11). The segment with a 10.5-bp repeat is expected to adopt a shape close to that of a planar circular molecule, whereas plasmids with a 10.0- or 11-bp repeat are expected to generate left- and right-handed superhelices, and this has been confirmed experimentally (9, 10). Plasmid pTH8 (3580 bp) contains two strong NR_I-binding sites and three weak binding sites (13); the two strong sites are located between 667 and 716 bp from the unique *Pvu* II restriction end of the linear form and are situated 100–149 bp upstream from the start of *glnA*p2 transcription (3, 14). The three weak binding sites are located downstream of the two strong binding sites in their normal position close to the promoter. Plasmids in supercoiled form were prepared by centrifugation in CsCl/ethidium bromide (EtdBr) gradients; linear plasmids were obtained by digestion with *Pvu* II. The relaxed DNA was prepared by treatment of the supercoiled form with pancreatic DNase I in the presence of a saturating amount of EtdBr.

Proteins. Mutant NR_I activator protein with Asp-54 → Glu and Ser-160 → Phe, called NTRC(D54E, S160F), fused with maltose-binding protein (MBP), was a generous gift of S. Kustu (University of California, Berkeley). This mutant NR_I binds to DNA in its unphosphorylated transcriptionally active form in the absence of ATP and NR_{II} (NTRB) protein (15, 16). In control experiments, 10 mM carbamoyl phosphate was added to the reaction mixture as a protein phosphorylating agent (11), without detectable change in NR_I binding to DNA (not shown). Ferritin was purchased from BRL.

Conditions for the Formation of DNA–NR_I Complexes. Reaction mixtures for EM contained 25 nM DNA and 290 nM NR_I mutant [NTRC(D54E, S160F)-MBP] in 50 mM Tris–acetate, pH 8/80 mM ammonium acetate/8 mM magnesium acetate/0.1 mM EDTA; for studies of the influence of increasing activator protein concentration (Fig. 4) the ratio of NR_I to DNA was increased up to 3 times—i.e., increasing the concentration of NR_I to 900 nM. (Plasmids have been linearized by digestion at the unique *Pvu* II restriction site.) For obtaining oriented molecules pTH8 has been cut with *Nhe* I restriction enzyme then end labeled with ferritin after incorporation of biotinylated dUTP (17) and recut with *Pvu* II. The reaction mixtures were incubated 10–20 min at 37°C before gel filtration on a Superose 6B (Pharmacia) column equilibrated with 50 mM Tris–acetate, pH 8/50 mM ammonium acetate/10 mM $MgCl_2$/0.1 mM EDTA. Gel filtration removed free NR_I from NR_I–DNA solution. Protein–DNA complexes eluted in the excluded volume were deposited onto pentylamine-activated carbon-coated grids.

EM. Grids were washed with 2% (wt/vol) uranyl acetate in water and dried on filter paper. Grids were observed by use of the annular dark-field illumination mode of a Zeiss CEM-902 electron microscope, as described (18). Images were recorded on Kodak electron image film at a magnification of either

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Abbreviations: EM, electron microscopy; MBP, maltose-binding protein.

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×50,000 or ×85,000. A Dage site-intensified tube camera fitted to the electron microscope allowed images to be directly digitized and analyzed on a Kontron image analyzer. An average of 50–100 molecules (Figs. 1 and 3) were digitized and analyzed to generate each DNA contour-length measurement and determine NR_I position on DNA.

RESULTS

To obtain more information about the mechanism of transcriptional activation by NR_I (NTRC), we inserted the sequence-induced supercoiled (spiral) segment $(CA_5TGCC)_{56}$ into DNA carrying the *glnA*p2 promoter, from which the specific NR_I-binding sequences had been deleted (pSB10). Cryo-EM (9, 10), transmission EM (B.R., E. Niedochodowiez, S.B., and G.B., unpublished work), and acrylamide gel electrophoresis confirm that the repetitive A tract adopts a plectonemic spiral structure. The discovery of the transcriptionally very active double mutant NR_I [NTRC(D54E, S160F)] (15, 16) allowed us to use this unphosphorylated form of NR_I protein. For the experiments that are presented below we used a fusion of NTRC(D54E, S160F) to MBP whose transcriptional activities, DNA binding, and ATPase activity are remarkably similar to those of the unfused NR_I double mutant (16).

Fig. 1 *Left* shows that NR_I binds selectively to a single site (*B–F*) corresponding to the superhelical insert (*A*) with high specificity. (The ends of the 560-bp spiral insert are located 816 and 1376 bp from one end of the linearized plasmid in *F*.) Analysis of the location of 110 complexes on the plasmid (pSB10) indicated that the bound NR_I activator is found in every case along the spiral insert only (Fig. 1 *Right*). The insert spans bp 816–1376, in good agreement with NR_I-covered positions 784–1408 of DNA. We have not found the activator protein complexed with any other part of plasmid pSB10 (Fig. 1 *Right*). Moreover, strong binding of NR_I to the spiral insert is suggested also by the use of gel filtration through Superose 6B, which preceded EM visualization (see *Materials and Methods*). The complex of NR_I with the supercoiled plasmid was invariably seen as an apical loop (Fig. 1 *Left, B* and *C*), which suggests that the spiral DNA is localized in apices, as was also observed for curved DNA in a superhelical plasmid (19). The presence of the activator protein in bent DNA was observed in complexes of NR_I with the plasmid in the relaxed (Fig. 1 *Left D*) or linear (Fig. 1 *Left F*; see also Fig. 4 *A* and *C*) forms, in good agreement with a previous observation (20); this may lead to DNA wrapping around NR_I (Figs. 1 *Left F* and 2 *D, E,* and *H*). Both observations suggest that NR_I is a DNA-bending protein which binds selectively to spiral DNA and induces further pronounced bending (21).

We also examined the interaction of NR_I with plasmid pTH8, which carries a pair of specific NR_I-binding sites constituting the *glnA* natural enhancer. Fig. 2 shows that all the features of specific binding of NR_I to the inserted supercoiled segment are also found with the plasmid carrying the natural enhancer. The binding of the activator protein to the apex of the supercoiled plasmid (Fig. 2 *A–C*) is clearly observed, whereas relaxed (*D*) and linear (*E* and *F*) plasmids show selective location of the activator protein molecules on their

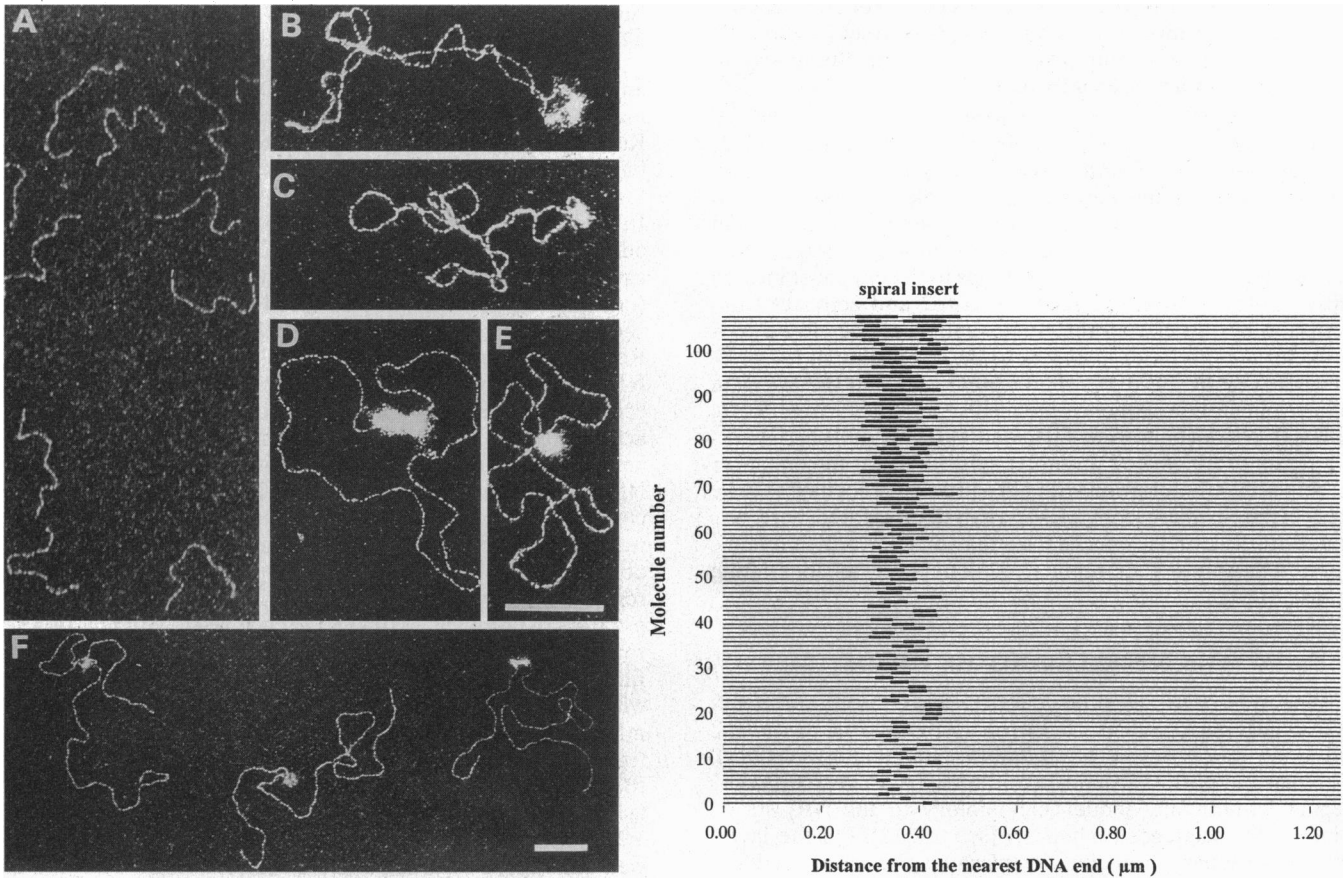

FIG. 1. (*Left*) NR_I [NTRC(D54E, S160F)-MBP] activator bound to the supercoiled (spiral) segment $(CA_5TGCC)_{56}$ inserted into plasmid pSB10 in its negatively supercoiled (*B* and *C*), relaxed (*D*), and linear (*E* and *F*) forms. (*A*) Insert alone, showing the spiral shape. (*B* and *C*) NR_I oligomers are localized at the apical loops of supercoiled DNA. (*D–F*) NR_I complexes with spiral segment of linear (*E* and *F*) and relaxed (*D*) plasmid (at lower protein-to-DNA ratio, 290 nM NR_I and 30 nM DNA). The plasmid pSB10 carries a spiral $(CA_5TGCC)_{56}$ tract inserted 48 bp upstream of the *glnA* promoter, but it lacks all natural NR_I-binding sites (11). The 560-bp spiral insert extends from 816 to 1376 bp from one end of the 3741-bp linearized plasmid. (Bars = 0.1 μm.) (*Right*) Selective binding of NR_I (thick bars) to the spiral insert in 110 molecules of plasmid pSB10 at high protein-to-DNA ratio (see Fig. 4).

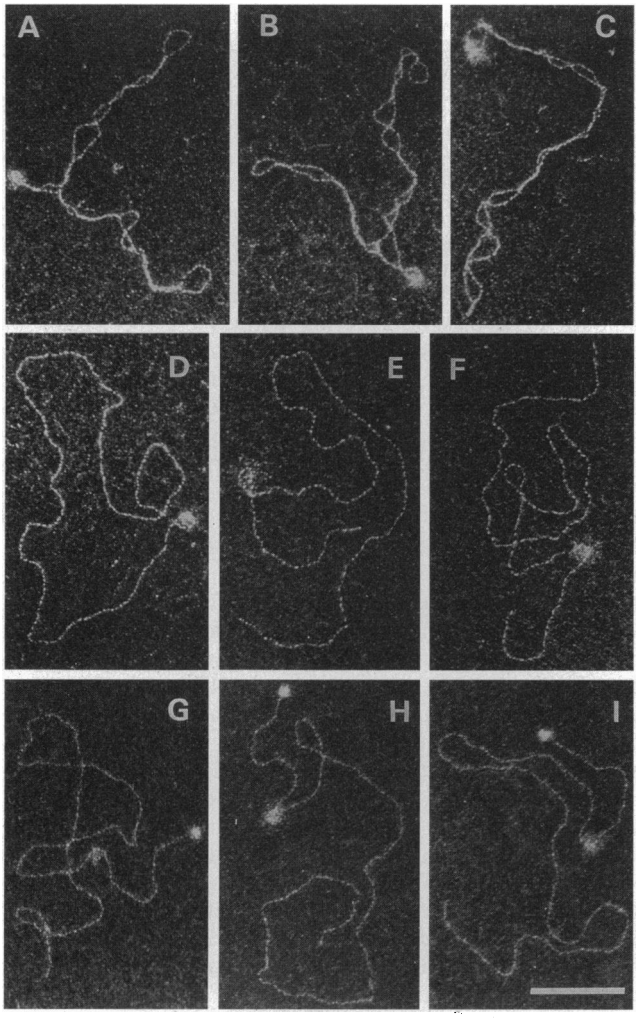

FIG. 2. NR_I [NTRC(D54E, S160F)-MBP] is binding to pTH8 (3580 bp) carrying the wild-type *glnA* promoter with two strong NR_I-binding sites (the natural enhancer) and three weak sites. (*A–C*) Supercoiled DNA, with NR_I molecules localized at apices. (*D*) Relaxed DNA. (*E* and *F*) *Pvu* II-linearized pTH8 DNA exhibiting the curvature that is induced by bound NR_I; NR_I-binding sites are located 690 bp from the closest end. (*G–I*) Oriented linear pTH8 uniquely end-labeled with ferritin after incorporation of biotinylated dUTP (17, 22). One can compare the more electron-dense ferritin with woolly but larger size NR_I. (Bar = 0.1 μm.)

specific binding sites; this is particularly clearly demonstrated with plasmid end-labeled with ferritin (Fig. 2 *G–I*). NR_I strongly curves DNA (Fig. 2*F*), and several pictures show DNA wrapping around the activator by one full turn (Fig. 2 *D, E,* and *H*). We conclude that selective strong binding of NR_I to the inserted spiral segment shows striking similarities with the activator binding to the enhancer, and that leads to bending and wrapping of the DNA around bound protein.

NR_I-induced bending is also confirmed by the EM analysis of the DNA curvature map of 60 molecules (Fig. 3) of plasmid pTH8, which carries two specific NR_I strong binding sites (enhancer). Upon NR_I binding, DNA appears strongly bent at the enhancer, considerably exceeding curvature fluctuations along the DNA chain (Fig. 3). This implies, as do the EM pictures in Fig. 2, that NR_I is a DNA-bending protein and supports the model of DNA wrapped around the activator. The experiments reported here support previous evidence that binding of NR_I to inserted supercoiled sequences may facilitate protein–protein interactions leading to tetramer and oligomer formation (12, 23). To obtain direct evidence for this view, we investigated NR_I–DNA complex formation with

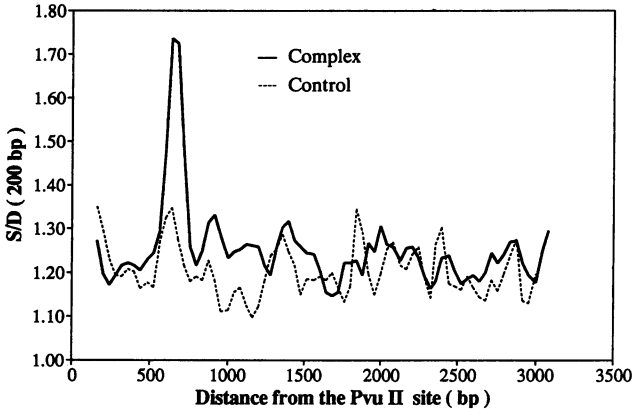

FIG. 3. EM curvature maps of the plasmid pTH8 linearized with *Pvu* II and complexed (solid line) or not (broken line) with NR_I. The curvature is defined by the ratio S/D of curvilinear distance (S) to the end-to-end length (D) of a 200-bp window moved by steps of 40 bp along the trajectory of the linear DNA (22). Sixty DNA molecules were analyzed. The position of the highest value of curvature corresponds to the location of bound NR_I and to binding sites (enhancer) obtained from measurements of complexes shown in Fig. 2 *G–I* of DNA end-labeled with ferritin. The locus of maximum curvature is 675 ± 40 bp from the *Pvu* II restriction site, whereas two strong NR_I-binding sites are situated between 667 and 716 bp from this site. A high increase in curvature is observed at this position after the binding of NR_I.

increasing amounts of protein. Fig. 4*A* shows that 90% of NR_I molecules are bound to the supercoiled segment (at low NR_I-to-DNA ratio), possibly as tetramer and oligomer. Moreover, Fig. 4 directly shows binding of different quantities of NR_I. The cooperativity of binding of NR_I, already shown (12, 23), is visualized in these EM images showing the single bound activator unit as possibly a tetramer and/or oligomer (Fig. 4*A*), whereas at higher protein concentrations the entire spiral insert is coated by bound NR_I multimers (Fig. 4 *B–D*).

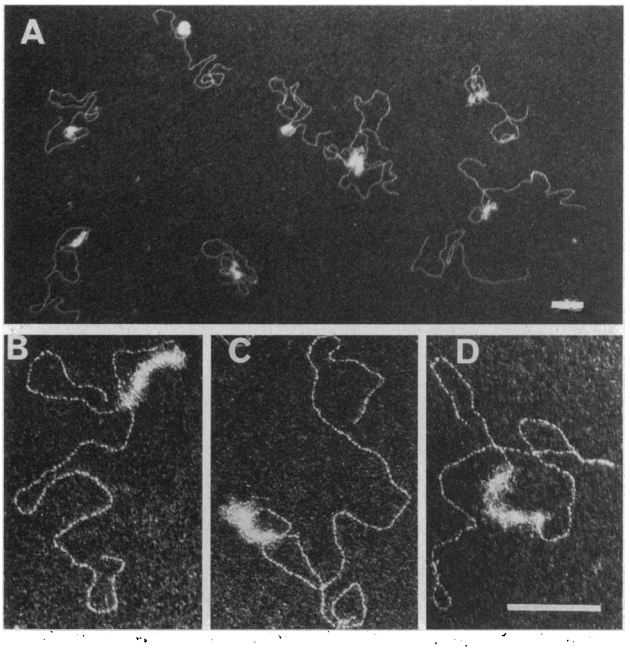

FIG. 4. Selective and cooperative binding of NR_I to the spiral segment of plasmid (pSB10). (*A*) Large field of plasmid DNA with bound NR_I (290 nM NR_I, 30 nM DNA). Ninety percent of pSB10 molecules remain complexed with NR_I after gel filtration on Superose 6B. (*B–D*) At protein-to-DNA ratio 3-fold higher than in *A* (900 nM NR_I, 30 nM linear DNA), the spiral insert is coated with NR_I oligomers; no other part of the plasmid shows bound activator protein. (Bars = 0.1 μm.)

We used two approaches to determine the size of bound protein. First, by direct measurements of the length of DNA (pSB10) covered by a single spot of bound NR_I we obtained the value of 80 bp (±10 bp), which was confirmed by independent measurements of the complex on the plasmid (pTH8) carrying the *glnA* natural enhancer (Fig. 2). This is to be compared with the minimal length of 49 bp obtained from NR_I footprinting and DNA sequencing of the *glnA* enhancer, which has two strong binding sites (14, 23). Consequently it suggests that NR_I binds to spiral DNA as two dimers to form a tetramer, similar to the situation with the *glnA* enhancer. Second, comparison of the size of the single spots of NR_I [NTRC(D54E, S160F)-MBP] ($M_r \approx 370{,}000$ for the tetramer) bound to DNA with that of ferritin ($M_r = 440{,}000$) and RNA polymerase ($M_r = 450{,}000$) (not shown) suggests that bound NR_I represents at least a tetramer and possibly a larger oligomer (Fig. 2 *G–I*).

To discern whether the inserted segment $(CA_5TGCC)_{56}$ binds NR_I by virtue of its tertiary structure or by some similarity with the strong binding sequence (enhancer) (consensus TGCACCA---TGGTGCA) (11, 12), we constructed plasmid pSB21E, which carries a spiral segment $(A_6TATATA_5TCTCT)_{59}$ without any resemblance to the enhancer sequence. As shown in Fig. 5, this spiral segment binds NR_I quite efficiently. To investigate the role of superhelical chirality on the binding of NR_I we constructed synthetic DNA tracts intrinsically bent with sequence repeats of 10 or 11 bp per helical turn which form left- and right-handed superhelices, respectively, whereas a repeat of 10.5 bp adopts the shape of a collapsed (nearly degenerate) spiral; these tracts were inserted into a vector carrying the *glnA*p2 promoter with deleted enhancer sequences (11). Electron micrographs indicate efficient NR_I binding to all of these sequence-induced spiral DNAs, regardless of their superhelical chirality (not shown). The absence of any observable effect of chirality of the DNA insert suggests that only the DNA curvature is an essential factor for efficient binding of NR_I and that the structural flexibility is too great for the existence of any discrimination between left- or right-handed superhelical DNA in NR_I complex formation.

DISCUSSION

We have previously shown strong binding of the activator protein NR_I to DNA containing a sequence-induced supercoil and that the binding generates activation of transcription (11). We now demonstrate, through EM visualization, high-affinity selective binding of NR_I exclusively to the spiral segment. In complexes of NR_I with relaxed or linear DNA, bending of the latter is observed, suggestive of DNA wrapping around NR_I. In supercoiled DNA, an apical loop localization of NR_I is clearly observed, which suggests that the spiral DNA is likewise localized in apices. Thus, NR_I is shown to be a DNA-bending protein, which may account for its high affinity for spiral DNA. The spiral DNA should also facilitate the suggested wrapping of DNA around NR_I. From the direct comparison of the NR_I binding to a plasmid carrying natural enhancer sites we conclude that the spiral segment substitutes for the enhancer by virtue of its high binding affinity and its ability to promote activator protein oligomerization, which explains its transcriptional stimulation and ATPase activation (11). It appears that the stoichiometry of NR_I binding to pTH8 and pSB10 is different; the images in Figs. 1 and 2 suggest a higher capacity for NR_I in the 560-bp supercoil than in a pair of strong NR_I-binding sites. This may explain the observed differences in the amount of bound NR_I despite the fact that the binding experiments with these two plasmids shown in Figs. 1 and 2 were performed under similar conditions. The functional consequence of this high binding affinity is difficult to interpret for a high ratio of activator protein to DNA, particularly in the absence of corresponding transcriptional data. At a significantly lower ratio of NR_I to DNA the plasmids containing superhelical inserts (e.g., pSB10 and pSB21) stimulate transcription as well as does the plasmid carrying the natural enhancer (11).

Finally, we show (Fig. 5) that the supercoil segment of pSB21E, $(A_6TATATA_5TCTCT)_{42}$, without any sequence homology to the enhancer binds NR_I quite efficiently, confirming that the tertiary structure is the essential cause for efficient binding to DNA and not the similarity of sequences with enhancer.

A main role of the enhancer is to maintain the activator protein in high local concentration near the promoter to increase the frequency with which it contacts σ^{54}-holoenzyme bound to the promoter, and we have shown that the superhelical insert entirely fulfills this requirement; in addition it promotes efficient cooperative binding to DNA and protein oligomerization leading to activation of transcription (12, 23). The ability of the superhelical DNA segment to act as enhancer may be generalized to other prokaryotic and eukaryotic transcriptional activators that bend DNA and act more efficiently as multimers.

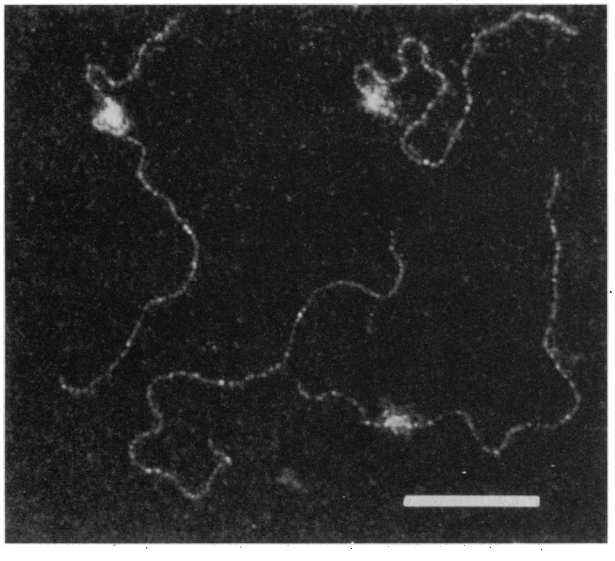

FIG. 5. NR_I binding to the supercoiled segment $(A_6TATATA_5TCTCT)_{59}$, which is without any sequence homology to natural enhancer. The majority of the DNA is complexed with NR_I. The EM preparations used 290 nM NRI and 30 nM DNA for complex formation. (Bar = 0.1 μm.)

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1. Banerji, J., Rusconi, S. & Schaffner, W. (1981) *Cell* **27**, 299–306.
2. Moreau, P., Hen, R., Wasylyk, B., Everett, R., Gaub, M. P. & Chambon, P. (1981) *Nucleic Acids Res.* **9**, 6047–6058.
3. Reitzer, L. J. & Magasanik, B. (1986) *Cell* **45**, 785–792.
4. Muller, M., Gerster, T. & Schaffner, W. (1988) *Eur. J. Biochem.* **176**, 485.
5. Ptashne, M. (1986) *Nature (London)* **322**, 697–701.
6. Magasanik, B. (1988) *Trends Biochem. Sci.* **13**, 475–479.
7. Kustu, S., North, A. K. & Weiss, D. S. (1991) *Trends Biochem. Sci.* **16**, 397–401.
8. Collado-Vides, J., Magasanik, B. & Gralla, J. D. (1991) *Microbiol. Rev.* **55**, 371–394.
9. Furrer, P., Bednar, J., Adrian, M., Stasiak, A., Dubochet, J., Révet, B., Brahms, S. & Brahms, G. (1992) in *Structure and*

Function: Nucleic Acids, Proceedings of the 7th Conversation in the Discipline Biomolecular Stereodynamics, State University of New York, Albany, eds. Sarma, R. & Sarma, M. (Adenine, New York), pp. 229–236.
10. Dubochet, J., Bednar, J., Furrer, P., Stasiak, A. Z. & Stasiak, A. (1994) *Struct. Biol.* **1,** 361–363.
11. Brahms, G., Brahms, S. & Magasanik, B. (1995) *J. Mol. Biol.* **246,** 35–42.
12. Weiss, V., Claverie-Martin, F. & Magasanik, B. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 5088–5092.
13. Hunt, T. P. & Magasanik, B. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 8453–8457.
14. Ninfa, A. J., Reitzer, L. J. & Magasanik, B. (1987) *Cell* **50,** 1039–1046.
15. Klose, K. E., Weiss, D. & Kustu, S. (1993) *J. Mol. Biol.* **232,** 67–78.
16. Klose, K. E., North, A. K., Stedman, K. M. & Kustu, S. (1994) *J. Mol. Biol.* **241,** 233–244.
17. Théveny, B. & Révet, B. (1987) *Nucleic Acids Res.* **15,** 947–958.
18. Révet, B., Sena, E. P. & Zarling, D. A. (1993) *J. Mol. Biol.* **232,** 779–791.
19. Laundon, C. H. & Griffith, J. D. (1988) *Cell* **52,** 545–549.
20. Su, W., Porter, S., Kustu, S. & Echols, H. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 5504–5508.
21. Crothers, D. M. (1994) *Science* **266,** 1819–1820.
22. Muzard, G., Théveny, B. & Révet, B. (1990) *EMBO J.* **9,** 1289–1298.
23. Porter, S., North, A. K., Wedel, A. B. & Kustu, S. (1993) *Genes Dev.* **7,** 2258–2273.
24. Herendeen, D. R., Kasavetis, G. A., Barry, J., Alberts, B. M. & Geiduschek, E. P. (1989) *Science* **245,** 952–958.