Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 9727–9731, October 1995
Biochemistry

DNA loops induced by cooperative binding of transcriptional activator proteins and preinitiation complexes

JÜRGEN C. BECKER*†, ABBAS NIKROO‡, THOMAS BRABLETZ§, AND RALPH A. REISFELD*

*Department of Immunology, IMM 13, The Scripps Research Institute, 10666 North Torrey Pines Road, La Jolla, CA 92037; ‡General Atomics, San Diego, CA 92186-9784; and §Department of Pathology, University of Erlangen-Nürnberg, Krankenhausstrasse 8-10, 91054 Erlangen, Germany

Communicated by M. Frederick Hawthorne, University of California, Los Angeles, CA, July 13, 1995

ABSTRACT **DNA conformational changes are essential for the assembly of multiprotein complexes that contact several DNA sequence elements. An approach based on atomic force microscopy was chosen to visualize specific protein–DNA interactions occurring on eukaryotic class II nuclear gene promoters. Here we report that binding of the transcription regulatory protein Jun to linearized plasmid DNA containing the consensus AP-1 binding site upstream of a class II gene promoter leads to bending of the DNA template. This binding of Jun was found to be essential for the formation of preinitiation complexes (PICs). The cooperative binding of Jun and PIC led to looping of DNA at the protein binding sites. These loops were not seen in the absence of either PICs, Jun, or the AP-1 binding site, suggesting a direct interaction between DNA-bound Jun homodimers and proteins bound to the core promoter. This direct visualization of functional transcriptional complexes confirms the theoretical predictions for the mode of gene regulation by trans-activating proteins.**

Transcriptional activation of eukaryotic genes during development or in response to extracellular signals involves the regulated assembly of multiprotein complexes on enhancers and promoters (1–3). Interaction between regulatory factors and the basal transcription machinery is often dependent on the relative positions of their respective binding sites on the DNA. Since the DNA helix is rather inflexible over short distances, interactions between proteins bound to closely spaced sites on DNA are constrained by the unfavorable increase in free energy caused by looping of the intervening DNA (4). However, the contact between proteins bound at nonadjacent sites on a DNA fragment can be facilitated by a bend in the DNA helix. Bending of DNA can be an intrinsic property of specific nucleotide sequences or can be induced by bound proteins (2, 3, 5–7).

The protooncogene c-*jun* encodes a protein, Jun, that is a member of the basic region–leucine zipper (bZIP) family. Circular dichroism spectroscopy has demonstrated that the DNA-binding domain of bZIP proteins undergoes a conformational transition to a structure of high α-helical content in the presence of the cognate DNA binding site (8). This α-helical region lies within the major groove of the B-DNA helix. According to thermodynamic principles, a conformational change in one molecule induced by interaction with a second molecule should cause a complementary conformational change in the latter (9). Indeed, DNA bending or increased DNA flexibility has been detected for several bZIP protein/DNA complexes by indirect methods—e.g., circular permutation or phasing analysis (7, 10, 11). However, of the currently available crystal structures only the GCN4/cAMP response element complex shows a significant deviation from straight B-DNA (12). This apparent discrepancy between the results of DNA bending experiments and crystal structures may indicate that the peptide fragments chosen for crystallization lack some of the residues needed for DNA bending, or that crystal packing forces may in some instances restrict DNA bending, or that the aberrant electrophoretic mobilities of these protein/DNA complexes do not result from a static bend in the DNA (13, 14).

Atomic force microscopy (AFM) has proven to be a powerful tool for investigating the structure of complex macromolecular assemblies in their native conformation (15–18); therefore, we used this technique for direct visualization of specific protein–DNA interactions.

MATERIALS AND METHODS

DNA Templates. The plasmids pBL-CAT4 and pTRE-CAT, containing the herpes simplex virus thymidine kinase promoter alone or together with an AP-1 binding site (tetradecanoylphorbol acetate response element, TRE) upstream of the chloramphenicol acetyltransferase (CAT) gene, have been described (19). These plasmids were linearized by digestion with the restriction enzyme *Sma* I, which cleaves at a site downstream of the CAT gene.

DNA-Binding Proteins. Recombinant Jun was produced as a glutathione *S*-transferase fusion protein. It was purified on glutathione-Sepharose, and the glutathione *S*-transferase portion was removed by thrombin digestion. Nuclear extracts for *in vitro* transcription and preinitiation complex (PIC) formation were prepared from Jurkat cells by the method of Dignam *et al.* (20). Biotin-conjugated double-stranded oligonucleotides containing two copies of the consensus AP-1 binding site were used for depletion of TRE binding activity. These binding reactions were conducted in 50 mM Hepes, pH 7.9/10% (vol/vol) glycerol/50 mM KCl/10 mM $MgCl_2$/1 mM EDTA/1 mM dithiothreitol/0.1% (wt/vol) poly(dI-dC) at 4°C for 30 min.

AFM Imaging of Protein–DNA Interactions. Protein–DNA binding reactions were conducted in 20 mM Hepes, pH 7.9/10 mM KCl/5 mM $MgCl_2$/1 mM EDTA/0.2 mM dithiothreitol in a final volume of 10 μl. For each reaction 500 ng of linearized plasmid and the indicated protein—either 50 ng of recombinant Jun, 500 ng of AP-1-depleted Jurkat nuclear extract, or both—were used. After a 15-min incubation at 4°C these samples were diluted 1:20 in water and 10 μl was deposited onto freshly cleaved, unmodified mica and dried under vacuum in a desiccator. Images were obtained in the height mode at scan rates between 4 and 8 Hz at 40% humidity with a Nanoscope III atomic force microscope (Digital Instruments, Santa Barbara, CA). Standard Si_3N_4 tips and typical forces of 5–20 nN were used.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: AFM, atomic force microscopy; bZIP, basic region–leucine zipper; CAT, chloramphenicol acetyltransferase; PIC, preinitiation complex; TBP, TATA-binding protein; TRE, tetradecanoylphorbol acetate response element.

†To whom reprint requests should be addressed.

***In Vitro* Transcription Assays.** *In vitro* transcription was performed in a volume of 25 μl containing 500 ng of linearized template DNA; 10 μg of nuclear extract; 12 mM Hepes (pH 7.9), 12% glycerol; 0.3 mM dithiothreitol, 0.12 mM EDTA; 60 mM KCl; 12 mM $MgCl_2$; 600 μM each ATP, CTP, and UTP; and 60 μM [α-^{32}P]GTP (10 Ci/mmol; 1 Ci = 37 GBq). Transcription complexes were preassembled in the absence of nucleotides for 30 min at 30°C. Following preassembly, nucleotides were added from a 5× stock solution, and transcription was allowed to proceed for 30 min at 30°C. *In vitro* transcription products were purified and analyzed by electrophoresis in 7 M urea/6% polyacrylamide sequencing gels and subsequently visualized with a Molecular Dynamics PhosphorImager.

RESULTS AND DISCUSSION

The effect of Jun homodimer binding to an ≈4-kb linearized CAT reporter construct (pTRE-CAT) containing a single AP-1 binding site upstream of the thymidine kinase promoter was imaged in air by AFM. Samples were prepared in a low-salt buffer, incubated for 15 min on ice, diluted 1:20 in water, and deposited on freshly cleaved mica. The control used was a CAT construct lacking the AP-1 binding site (pBL-CAT4) that was otherwise identical to pTRE-CAT. Images of pTRE-CAT in the absence of Jun showed straight filaments with sporadic bends (Fig. 1*A*). In a number of plasmids, globular structures were observed at the end of the filaments. These are likely to be residual restriction endonucleases, which were used to linearize the plasmids. Overdigestion by these restriction endonucleases or exposure to shearing forces created during sample preparation may explain the occasional occurrence of shorter filaments in the plasmid preparations. When Jun was present in the sample, protein/DNA complexes could clearly be seen (Fig. 2*A*). Jun homodimer appeared as a larger, symmetrical structure bound to the DNA strand. The DNA and the Jun/DNA complex were, on average, 28 and 68 nm wide and 1.4 nm and 12 nm high, respectively. This indicates strong tip convolution effects on the apparent widths of the molecules. Distinct bends in the DNA at the binding site of the protein were observed. Jun/DNA complexes could not be detected with the control plasmid, which lacks the binding site for Jun homodimers (Fig. 1*B*).

Statistical analyses of the distribution of the DNA bend angle observed in the Jun/DNA complexes were performed. Ninety-seven complexes were selected from six different samples. Lines were drawn through the axes of the DNA molecules on both sides of the protein/DNA complex, and the angle of their intersection (Φ) was measured (Fig. 2*A*). The bend angle (θ) is the supplement of the measured angle Φ to 180° ($\theta = 180° - \Phi$). The mean value for θ was found to be 36° (Fig. 2*B*). This value is consistent with the theoretical predictions obtained from computer-generated helical-axis trajectories for the DNA conformational changes upon binding of Jun homodimers to the AP-1 consensus site (21). It should be taken into consideration that the magnitude of the bending angle may be underestimated due to the possible stretching of the DNA molecules induced by forces involved during the drying process after deposition (22). However, control experiments using mica chemically modified with Mg^{2+}, which has been shown to increase the binding force between mica and DNA and thereby should decrease the DNA stretching during drying (23, 24), showed a comparable distribution of bending angles after binding of Jun (data not shown). Furthermore, if the observed bend-angle distribution is to be accounted for by random thermal fluctuations, these should be isotropic and thus centered at a mean value of $\theta = 0°$. Comparison of the observed bend-angle distribution with such a theoretical distribution by the Kolmogorov–Smirnov test resulted in a P value of <0.005.

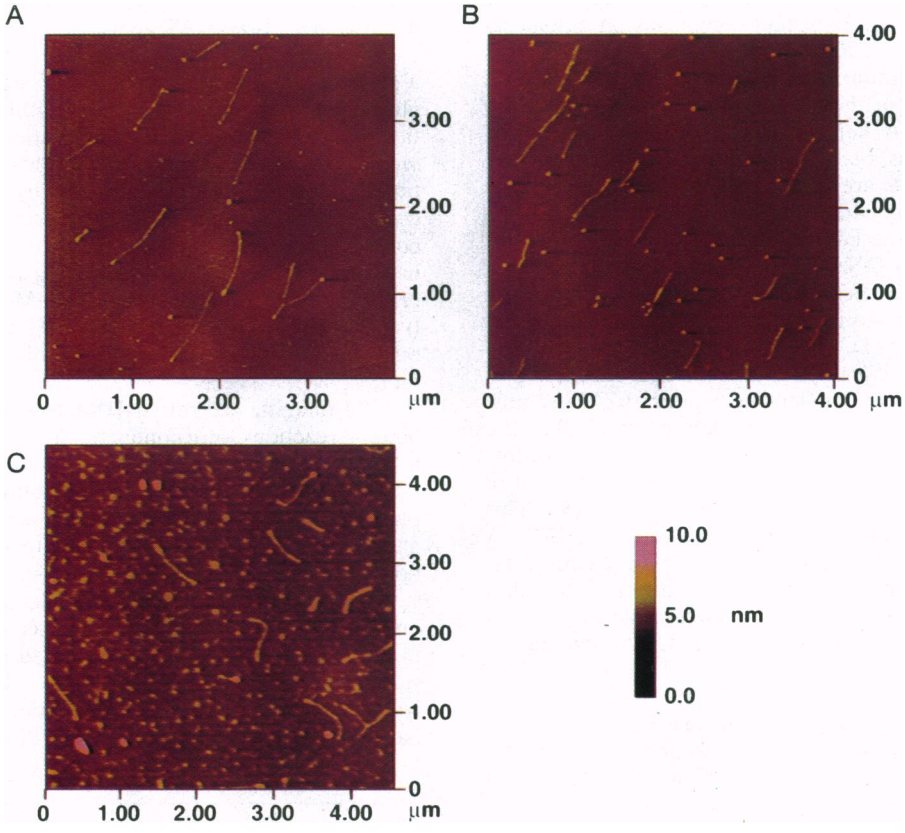

FIG. 1. AFM images of pTRE-CAT alone (*A*) and pBL-CAT in the presence of either recombinant Jun (*B*) or Jurkat nuclear extract (*C*). For each reaction 500 ng of linearized plasmid and either 50 ng of recombinant Jun or 500 ng of AP-1-depleted Jurkat nuclear extract were used. Images were obtained in the height mode at scan rates of 4–8 Hz with standard Si_3N_4 tips.

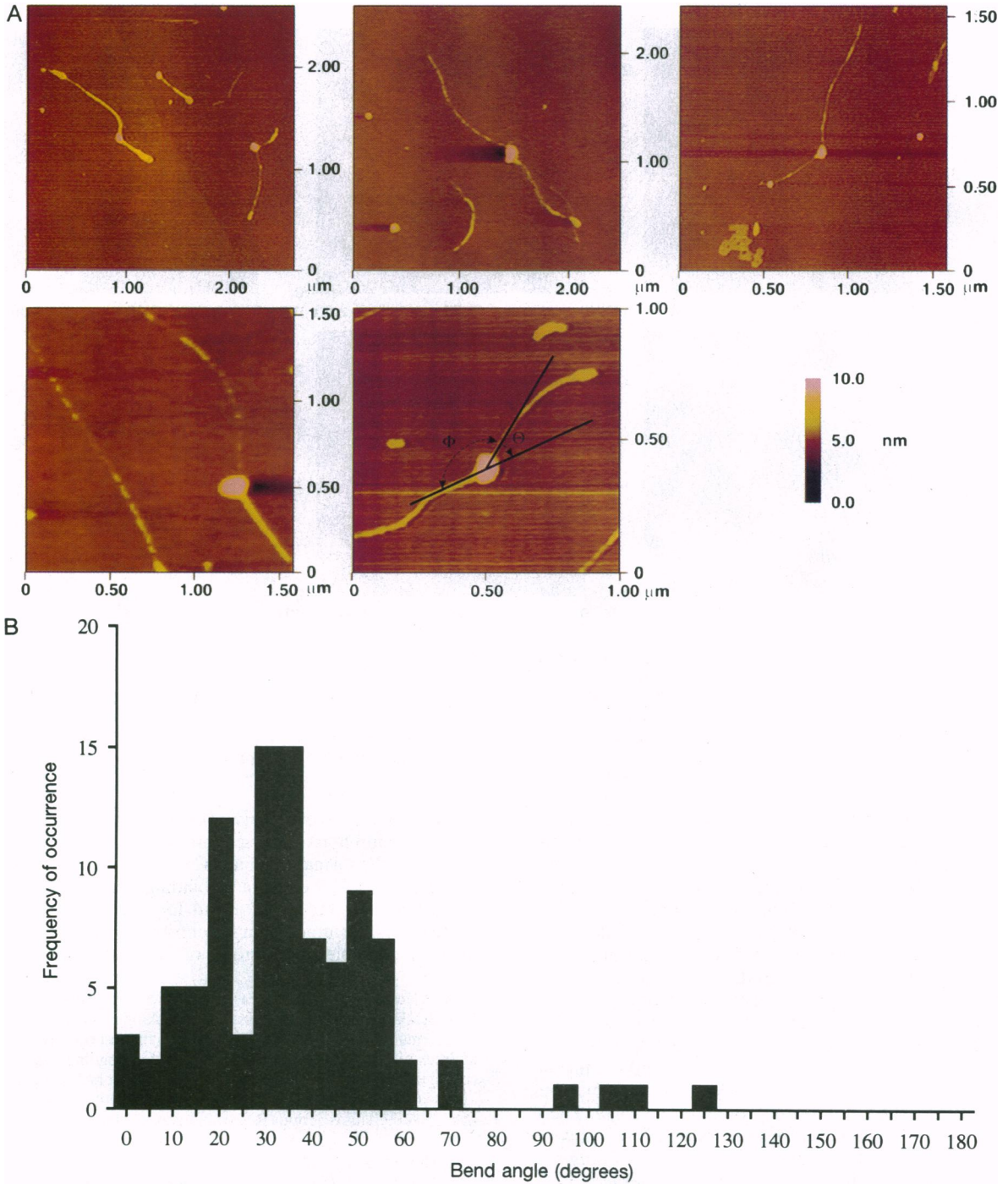

FIG. 2. Bending of pTRE-CAT upon binding of Jun. (*A*) AFM images of pTRE-CAT in the presence of recombinant Jun. For each reaction 500 ng of plasmid and 50 ng of recombinant Jun were used. Images were obtained in the height mode at scan rates of 4–8 Hz with standard Si_3N_4 tips. The DNA and the Jun/DNA complex were, on average, 28 and 68 nm wide and 1.4 nm and 12 nm high, respectively. This indicates strong tip convolution effects on the apparent widths of the molecules. (*B*) Frequency of occurrence of Jun/DNA complexes with various bend angles (n = 97). Only complexes that were unobstructed were chosen. The bend angle, θ, is the supplement of the measured angle Φ to 180° ($\theta = 180° - \Phi$).

One biological role for the bending of DNA upon binding of transcriptional activator proteins is to facilitate the interaction of such proteins with the PIC (2, 3, 25). Previous reports of direct and selective binding of a number of trans-activating proteins either to the TATA-binding protein (TBP) or to TBP-associated factors (26–28) further support the hypothesis that looping of intervening DNA is a necessary event in transcriptional regulation. The most direct evidence supporting this hypothesis was provided by electron microscopy showing DNA looping induced by cooperative binding of either the λ repressor (29) or the *Xenopus* high-mobility group (HMG)-box transcription factor, xUBF (30). However, direct evidence for DNA looping in the case of multiprotein complexes binding to class II nuclear gene promoters has remained elusive. To visualize such multiprotein complexes bound to DNA, we isolated nuclear extracts from Jurkat T-cells and depleted them of AP-1 binding activity. These extracts contained all proteins necessary for assembly of PICs and induction of basal transcription *in vitro*. The functional capacity of these extracts was confirmed by *in vitro* transcription assays (Fig. 3*A*). In the

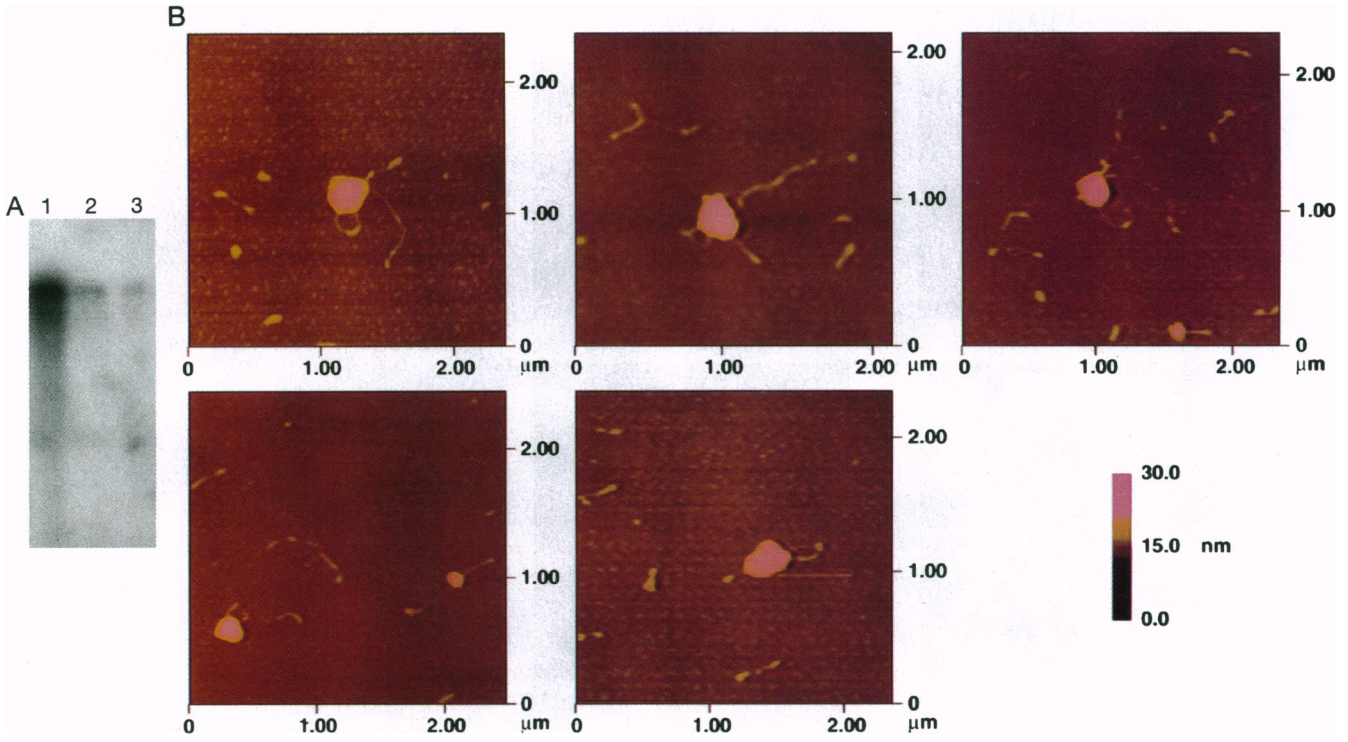

FIG. 3. Formation of functional transcription complexes at the promoter region of pTRE-CAT. (*A*) *In vitro* transcription from pTRE-CAT (lanes 1 and 2) and pBL-CAT (lane 3) induced by AP-1-depleted Jurkat nuclear extract alone (lane 2) or after reconstitution of the extract with Jun (lanes 1 and 3). (*B*) AFM images of the looping of pTRE-CAT upon binding of Jun in the presence of Jurkat nuclear extracts. For each reaction 500 ng of plasmid and 500 ng of AP-1-depleted Jurkat nuclear extract were used; where indicated, 50 ng of recombinant Jun was added. Images were obtained in the height mode at scan rates of 4–8 Hz at 40% humidity. Standard Si_3N_4 tips and typical forces of 5–20 nN were used. The Jun/PIC/DNA complex was, on average, 150 nm wide and 20 nm high. In comparison, the DNA and the Jun/DNA complex were, on average, 28 and 68 nm wide and 1.4 nm and 12 nm high, respectively.

presence of Jun, they induced significant transcription from pTRE-CAT, but only a very low basal transcription was observed when the extracts were used alone. With the plasmid pBL-CAT, which lacks the AP-1 binding site, the same extracts produced only basal transcription in both the presence and the absence of Jun. AFM images of pTRE-CAT incubated with Jun and these nuclear extracts showed the binding of protein complexes to the DNA that were significantly larger than those observed when Jun homodimers were used alone (Fig. 3*B*). The Jun/PIC/DNA complex was, on average, 150 nm wide and 20 nm high. In comparison, the DNA and the Jun/DNA complexes were, on average, 28 and 68 nm wide and 1.4 nm and 12 nm high, respectively. Binding of these protein complexes induced looping of the DNA at the protein binding site. These loops comprised a few hundred base pairs of DNA and were not seen when Jun, nuclear extract, or the AP-1 binding site was omitted. These findings suggest a direct interaction between DNA-bound Jun homodimers and proteins bound to the core promoter. This looping of DNA is likely to be facilitated not only by bending of the DNA by binding of Jun but also, as previously reported, by DNA bending induced upon binding of TBP or RNA polymerase (5, 17). When the control plasmid lacking the AP-1 binding site was used under the same conditions, the samples not only lacked the DNA loops but also displayed no obvious binding of proteins to DNA (Fig. 1*C*). This finding suggests that binding of Jun to pTRE-CAT may either increase the recruitment of the basal transcription complex to the core promoter or stabilize the formed complex. This observation is in agreement with studies which indicate that trans-activating factors stimulate transcription by increasing the number of functional PICs (25, 27, 31).

The ability of the atomic force microscope to visualize individual DNA molecules makes it a valuable tool for investigating DNA conformations at the molecular level. This instrument made it possible to obtain visual evidence that DNA is bent upon binding of Jun homodimers. Moreover, we were able to demonstrate that the formation of a PIC in the presence of Jun leads to looping of DNA at the protein binding sites. We anticipate that AFM will continue to be useful for visualizing the effects of protein/DNA interactions and contribute to a better understanding of the complex mechanisms of transcriptional regulation.

We thank Drs. Deborah A. Sweet (The Scripps Research Institute) and Paul Staskus (General Atomics) for stimulating discussions and critical comments during the preparation of the manuscript. We express our appreciation to Dr. Eva-Bettina Bröcker for ongoing support and encouragement. J.C.B. is a fellow of the Deutsche Forschungsgemeinschaft. This work was supported in part by National Institutes of Health Grant CA42508-09. This is Scripps Research Institute manuscript no. 9018-Imm.

1. Buratowski, S. (1994) *Cell* **77,** 1–3.
2. Ptashne, M. (1986) *Nature (London)* **322,** 697–701.
3. Tjian, R. & Maniatis, T. (1994) *Cell* **77,** 5–8.
4. Wang, J. C. & Giaever, G. N. (1988) *Science* **240,** 300–304.
5. Kim, Y., Geiger, J. H., Hahn, S. & Sigler, P. B. (1993) *Nature (London)* **365,** 512–520.
6. Giese, K., Cox, J. & Grosschedl, R. (1992) *Cell* **69,** 185–195.
7. Ellenberger, T. (1994) *Curr. Opin. Struct. Biol.* **4,** 12–21.
8. Patel, L., Abate, C. & Curran, T. (1990) *Nature (London)* **347,** 572–574.
9. Travers, A. A. & Klug, A. (1987) *Nature (London)* **327,** 280–281.
10. Kerppola, T. K. & Curran, T. (1991) *Cell* **66,** 317–326.
11. Kerppola, T. K. & Curran, T. (1991) *Science* **254,** 1210–1214.
12. König, P. & Richmond, T. J. (1993) *J. Mol. Biol.* **233,** 139–154.
13. Mills, J. B., Cooper, J. P. & Hagerman, P. J. (1994) *Biochemistry* **33,** 1797–1803.
14. Kahn, J. D., Yun, E. & Crothers, D. M. (1994) *Nature (London)* **368,** 163–166.
15. Guthold, M., Bezanilla, M., Erie, D. A., Jenkins, B., Hansma, H. G. & Bustamente, C. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 12927–12931.

16. Erie, D. A., Yang, G., Schultz, H. C. & Bustamente, C. (1994) *Science* **266,** 1562–1566.
17. Rees, W. A., Keller, R. W., Vesenka, J. P., Yang, G. & Bustamente, C. (1993) *Science* **260,** 1646–1649.
18. Hansma, H. G., Vesenka, J., Siegerist, C., Kelderman, G., Morett, H., Sinsheimer, R. L., Elings, V., Bustamente, C. & Hansama, P. K. (1992) *Science* **256,** 1180–1184.
19. Becker, J. C., Brabletz, T., Kirchner, T., Conrad, C. T., Bröcker, E. B. & Reisfeld, R. A. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 2375–2378.
20. Dignam, J. D., Lebovitz, R. M. & Roeder, R. G. (1983) *Nucleic Acids Res.* **11,** 1475–1489.
21. Harrington, R. E. & Winicov, L. (1994) *Prog. Nucleic Acid Res. Mol. Biol.* **47,** 195–270.
22. Thundat, T., Allison, D. P., Warmack, R. J., Brown, G. M., Jacobson, K. B., Schrick, J. J. & Ferrell, T. L. (1992) *Scanning Microsc.* **6,** 911–918.
23. Allison, D. P., Bottomley, L. A., Thundat, T., Brown, G. M., Woychik, R. P., Schrick, J. J., Jacobson, K. B. & Warmack, R. J. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 10129–10133.
24. Lyubchenko, Y. L., Shlyakhtenko, L., Harrington, R., Oden, P. & Lindsay, S. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 2137–2140.
25. Roberts, S. G. E. & Green, M. R. (1994) *Nature (London)* **371,** 717–720.
26. Gill, G., Pascal, E., Tseng, Z. H. & Tjian, R. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 192–196.
27. Lin, Y.-S. & Green, M. R. (1991) *Cell* **64,** 971–981.
28. Stringer, K. F., Ingles, C. J. & Greenblatt, J. (1990) *Nature (London)* **345,** 783–786.
29. Griffith, J., Hochschild, A. & Ptashne, M. (1986) *Nature (London)* **322,** 750–752.
30. Bazett-Jones, D. P., Leblanc, B., Herfort, M. & Moss, T. (1994) *Science* **264,** 1134–1137.
31. Klein, C. & Struhl, K. (1994) *Science* **266,** 280–282.