

# Transcriptional Activation *via* DNA-looping: Visualization of Intermediates in the Activation Pathway of *E. coli* RNA Polymerase $\cdot\sigma^{54}$ Holoenzyme by Scanning Force Microscopy

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Scanning force microscopy (SFM) has been used to study transcriptional activation of *Escherichia coli* RNA polymerase  $\cdot\sigma^{54}$  (RNAP  $\cdot\sigma^{54}$ ) at the *glnA* promoter by the constitutive mutant NtrC<sup>D54E,S160F</sup> of the NtrC Protein (nitrogen regulatory protein C). DNA-protein complexes were deposited on mica and images were recorded in air. The DNA template was a 726 bp linear fragment with two NtrC binding sites located at the end and about 460 bp away from the RNAP  $\cdot\sigma^{54}$  *glnA* promoter. By choosing appropriate conditions the structure of various intermediates in the transcription process could be visualized and analyzed: (1) different multimeric complexes of NtrC<sup>D54E,S160F</sup> dimers bound to the DNA template; (2) the closed complex of RNAP  $\cdot\sigma^{54}$  at the *glnA* promoter; (3) association between DNA bound RNAP  $\cdot\sigma^{54}$  and NtrC<sup>D54E,S160F</sup> with the intervening DNA looped out; and (4) the activated open promoter complex of RNAP  $\cdot\sigma^{54}$ . Measurements of the DNA bending angle of RNAP  $\cdot\sigma^{54}$  closed promoter complexes yielded an apparent bending angle of 49(±24)°. Under conditions that allowed the formation of the open promoter complex, the distribution of bending angles displayed two peaks at 50(±24)° and 114(±18)°, suggesting that the transition from the RNAP  $\cdot\sigma^{54}$  closed complex to the open complex is accompanied by an increase of the DNA bending angle.

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## Introduction

The vast majority of promoters in enteric bacteria are recognized by RNA polymerase complexed with the  $\sigma^{70}$  sigma factor (RNAP  $\cdot\sigma^{70}$  holoenzyme). Binding sites for proteins that regulate transcription from these promoters are found at positions close enough to permit direct contact between the regulator and RNAP  $\cdot\sigma^{70}$  at the promoter (Collado-Vides *et al.*, 1991). In contrast, transcription by RNA polymerase  $\cdot\sigma^{54}$  (RNAP  $\cdot\sigma^{54}$ ) holoenzyme requires a specific promoter consensus sequence and is controlled from activators whose distal location requires looping of the intervening

DNA to interact with RNAP  $\cdot\sigma^{54}$  at the promoter (Collado-Vides *et al.*, 1991; North *et al.*, 1993; Reitzer & Magasanik, 1986). Transcriptional activation from distant DNA sequences that are called enhancers or upstream elements was first described in eukaryotes. Because of their apparent similarity, the DNA sequences involved in the activation of the RNAP  $\cdot\sigma^{54}$  have been termed "prokaryotic enhancers" (Kustu *et al.*, 1985). This denomination appears justified since various other aspects of RNAP  $\cdot\sigma^{54}$  transcription resemble eukaryotic transcription by RNA polymerase II (Atkinson & Ninfa, 1994; Gralla, 1991; Weiss *et al.*, 192a): (1) the existence of a low basal level of transcription not easily detectable under physiological conditions; (2) the requirement of ATP hydrolysis for open complex formation; and (3) the existence of sequence motifs like acidic regions, a glutamine-rich sequence and hydrophobic repeats in  $\sigma^{54}$  that

Abbreviations used: SFM, scanning force microscopy; RNAP, RNA polymerase; NtrC, nitrogen regulatory protein C; IPTG, isopropyl  $\beta$ -D-thiogalactopyranoside; PMSF, phenylmethylsulfonyl fluoride.

are believed to mediate protein-protein interactions with prokaryotic transcriptional activators and are also found in eukaryotic activators (Gralla, 1991; Sasse-Dwight & Gralla, 1990).

This paper describes the use of scanning force microscopy (SFM) (reviewed by Bustamante *et al.*, 1994, 1993; Bustamante & Rivetti, 1996; Hansma & Hoh, 1994) to follow the transcription activation process of RNAP· $\sigma^{54}$  by the constitutive mutant NtrC<sup>D54E,S160F</sup> of the activator protein NtrC (nitrogen regulatory protein C, also designated as NR<sub>I</sub>). Scanning force microscopy (SFM) has been used successfully to image biological samples in air or in liquid without use of external means of contrast. In various studies it has been demonstrated that this technique can provide new insight in the field of transcription (Guthold *et al.*, 1994; Kasas *et al.*, 1997; Rees *et al.*, 1993; Wyman *et al.*, 1997). At present, the resolution of the SFM is comparable to conventional electron microscopy which has also been used to study NtrC and/or RNAP· $\sigma^{54}$  (Révet *et al.*, 1995; Su *et al.*, 1990). However, SFM is a topographic technique so that additional information is also encoded in the height of the sample. Furthermore, it has been shown recently that SFM images of DNA and DNA-protein complexes are a faithful representation of their solution structure (Rivetti *et al.*, 1996; C. Walker & C. Bustamante, unpublished results). In particular, the deposition process is very gentle and slow, so that DNA and DNA-protein samples can equilibrate on the substrate. Complexes on the surface therefore assume a lowest free energy state similar to that in solution. The system studied here consists of core RNA polymerase and  $\sigma^{54}$  from *Escherichia coli* and the constitutive NtrC<sup>D54E,S160F</sup> mutant from *Salmonella* bound to a linear EcoRI-PstI fragment from pJES534 that has two strong binding sites for NtrC and the *glnA* promoter (Klose *et al.*, 1993; Porter *et al.*, 1993; Figure 1). Both *E. coli*  $\sigma^{54}$  (Hsieh *et al.*, 1994; Sasse-Dwight & Gralla, 1990; Tintut *et al.*, 1994; Wang *et al.*, 1995; Wong & Gralla, 1992; Wong *et al.*, 1994) and NtrC from *Salmonella* (Flashner *et al.*, 1995; Klose *et al.*, 1994, 1993; North *et al.*, 1996) have been thoroughly characterized by mutation analysis. To activate transcription, wild-

type NtrC must be phosphorylated *in vivo* by the NtrB protein (nitrogen regulatory protein B, also designated as NR<sub>II</sub>) at Asp54. In contrast, the constitutively active double mutant NtrC<sup>D54E,S160F</sup> used here does not require phosphorylation to activate transcription by RNAP· $\sigma^{54}$  (Klose *et al.*, 1993).

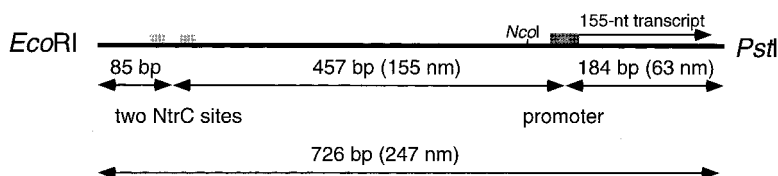
The formation of open promoter complexes with RNAP· $\sigma^{54}$  requires the presence of both NtrC<sup>D54E,S160F</sup> and ATP. To initiate transcription CTP and GTP are added to the reaction and stalled elongation complexes with a 155 nt U-less transcript are formed. Hence, in this system various stages of the transcription activation process can be separated and by sequentially adding to the template RNAP· $\sigma^{54}$ , NtrC<sup>D54E,S160F</sup>, ATP, or CTP and GTP, the following species were imaged: (1) multimeric complexes of NtrC<sup>D54E,S160F</sup> dimers at the two NtrC sites at the EcoRI end of the template; (2) closed complexes of RNAP· $\sigma^{54}$  bound specifically to the *glnA* promoter with a characteristic DNA bending angle; (3) a looping intermediate between DNA bound NtrC<sup>D54E,S160F</sup> and RNAP· $\sigma^{54}$ , that appeared in the presence of ATP; and (4) the open complex of RNAP· $\sigma^{54}$  at the *glnA* promoter that was characterized by a change in the DNA bending angle as compared to the closed complex.

## Results

### The RNAP· $\sigma^{54}$ /NtrC<sup>D54E,S160F</sup> transcription system

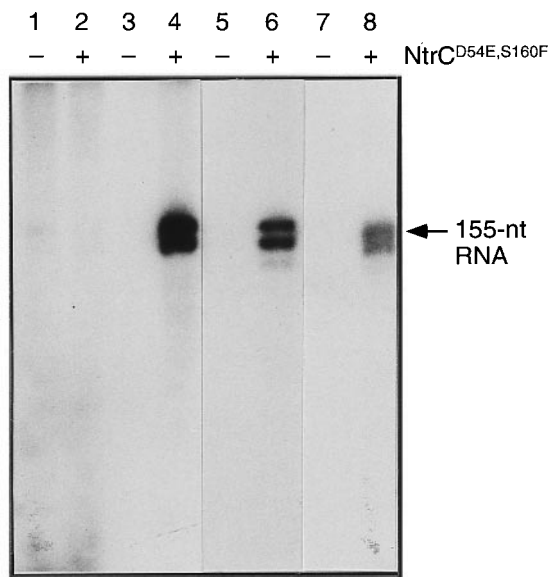
Transcription of RNAP· $\sigma^{54}$  on the pJES534 template has been studied by Kustu and co-workers (Klose *et al.*, 1993; Porter *et al.*, 1993), who also identified the constitutively active NtrC<sup>D54E,S160F</sup> mutant (Klose *et al.*, 1993). The linear template derived from their pJES534 template by EcoRI and PstI digestion is shown in Figure 1. To facilitate the protein purification procedure the genes for  $\sigma^{54}$  and NtrC<sup>D54E,S160F</sup> were cloned in pET15b from Novagen and over-expressed with a N-terminal His-tag (see Materials and Methods).

In Figure 2 the activation of RNAP· $\sigma^{54}$  transcription by NtrC<sup>D54E,S160F</sup> is demonstrated for the



**Figure 1.** DNA template used for SFM. The plasmid pJES534 was digested with EcoRI and PstI (Klose *et al.*, 1993; Porter *et al.*, 1993). The resulting 726 bp (247 nm) long fragment has two identical strong NtrC binding sites, located at the EcoRI end of the fragment and the *glnA*

promoter located 60 nm away from the PstI end. The two NtrC binding sites (in bold) have the sequence: **TGCAC-TAAAATGGTGCA** TAATGTTAACATTAA **TGCACTAAAATGGTGCA**. The center-to-center distance between the two NtrC binding sites and the *glnA* promoter is 457 bp (155 nm). RNAP· $\sigma^{54}$  produces a 155 nt U-less template from the *glnA* promoter upon incubation with ATP, CTP, GTP. For the transcription experiments (Figure 2) pJES534 linearized with NcoI and the whole pJES534 plasmid at native superhelical density were used in addition to the EcoRI and PstI fragment. The NcoI site is located between the promoter and the NtrC binding sites and results in a 3500 bp linear fragment on which the NtrC binding sites and the *glnA* promoter are separated by about 3000 bp.



**Figure 2.** *In vitro* transcription from different templates. Polyacrylamide gel analysis of single round transcription assays (see Materials and Methods). Different templates were compared in the absence (–) or presence (+) of NtrC<sup>D54E,S160F</sup>. Lanes 1 and 2, transcription from the linear *EcoRI*-*PstI* fragment with RNAP· $\sigma^{70}$ . Lanes 3 and 4, transcription from pJES534 at native superhelical density with RNAP· $\sigma^{54}$ . Lanes 5 and 6, transcription from the linear *EcoRI*-*PstI* fragment with RNAP· $\sigma^{54}$ . Lanes 7 and 8, transcription from *NcoI* linearized pJES534 with RNAP· $\sigma^{54}$ . A 2 to 3 bp shorter product appeared in addition to the 155 nt full length transcript with both the superhelical plasmid as well as with the linear templates, which is likely to be related to a second initiation site (Klose *et al.*, 1993; Wedel & Kustu, 1995).

protein preparations (His-tagged  $\sigma^{54}$ , His-tagged NtrC<sup>D54E,S160F</sup> and core RNA polymerase) and the DNA template used here. Lane 6 shows transcription with the 726 bp linear template (Figure 1), whereas in lane 4 the complete pJES534 plasmid at native superhelicity and in lane 8 the *NcoI* linearized plasmid have been used. The *NcoI* site is located between the promoter and the tandem NtrC binding sites, and cutting results in a separation distance of about 3000 bp between enhancer and promoter as opposed to about 460 bp for the two other templates (Figure 1). The amount of transcript produced was highest with the superhelical template, the 726 bp linear fragment being the next most effective, and the *NcoI* linearized plasmid yielding the lowest amount of transcript.

In addition to the expected 155 nt full-length transcript, a product shorter by 2 to 3 bp appeared with both the superhelical plasmid and the linear templates. This product is probably transcribed from a slightly different initiation site, which has been observed with the same promoter under similar conditions (Klose *et al.*, 1993; Wedel & Kustu, 1995). No specific transcript was detected in the ab-

sence of NtrC<sup>D54E,S160F</sup> (Figure 2, lanes 3, 5, and 7) or when RNAP· $\sigma^{54}$  was replaced with RNAP· $\sigma^{70}$  (Figure 2, lanes 1 and 2).

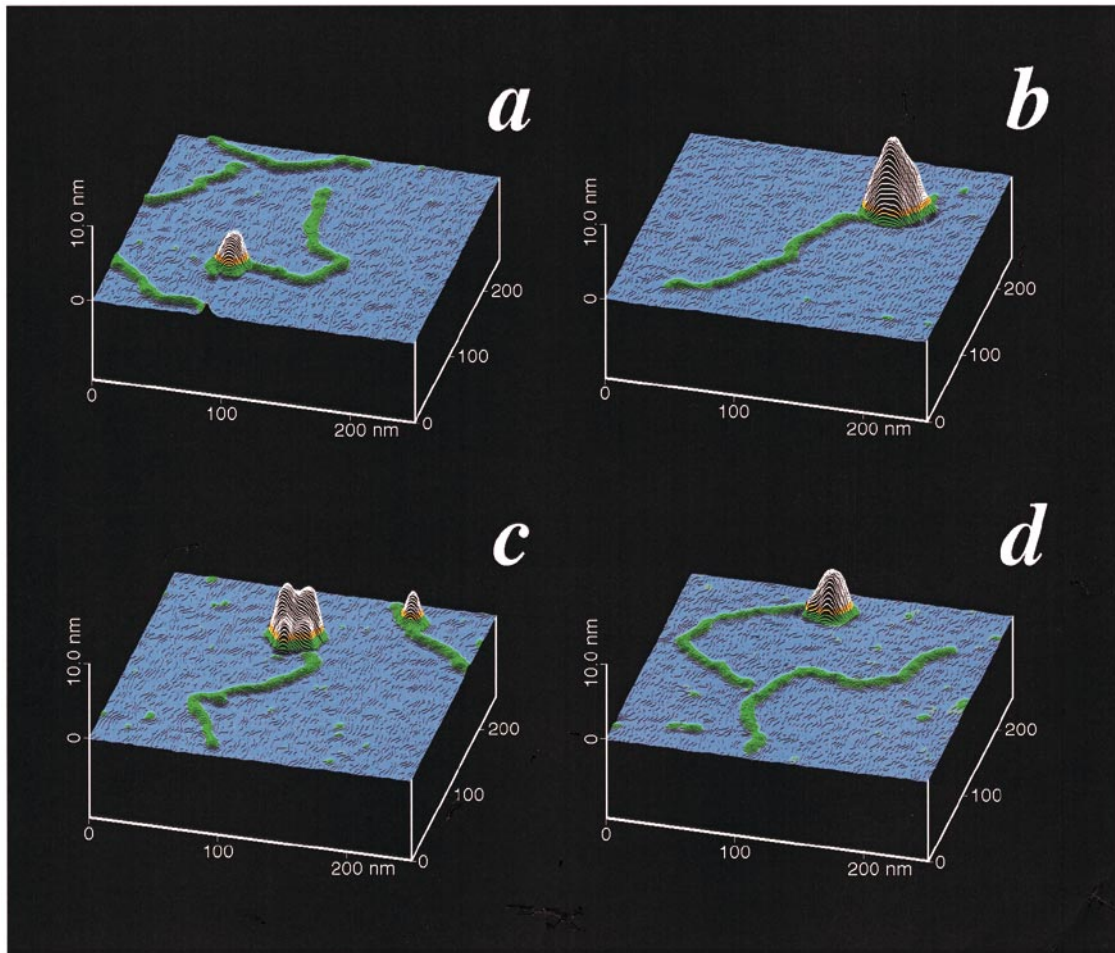
### Binding of NtrC<sup>D54E,S160F</sup>

The two NtrC-binding sites are located at the *EcoRI* end of the fragment and are separated from the *glnA* promoter by about 460 bp or 155 nm (center-to-center distance of the two sites) as shown in Figure 1. Upon incubation of the DNA template with NtrC<sup>D54E,S160F</sup>, DNA-protein complexes positioned at one end of the fragment were observed, as expected from the location of the tandem sites. Under the conditions of the experiment, more than 90% of the DNA fragments had NtrC<sup>D54E,S160F</sup> proteins bound at one end of the template (data not shown). Figure 3 displays 250 nm × 250 nm images of four different NtrC<sup>D54E,S160F</sup>-DNA complexes that were taken from the same 2  $\mu$ m × 2  $\mu$ m scan and can thus be directly compared in terms of size and shape. It is apparent that the four complexes displayed all have different sizes.

It has been shown (Klose *et al.*, 1994; Porter *et al.*, 1993) that NtrC binds as a dimer ( $M_r$  104 kDa) to each of the two DNA sites that constitute the strong enhancer. Thus, one would expect in the simplest case to find molecules that have one or two NtrC<sup>D54E,S160F</sup> dimers bound. The appearance of additional species with a size similar to that of RNAP· $\sigma^{54}$  (450 kDa), or even larger (see below), suggests that, in addition to the two DNA bound NtrC<sup>D54E,S160F</sup> dimers, two or more additional NtrC<sup>D54E,S160F</sup> dimers can be bound *via* protein-protein interactions. A detailed study of the association states of wild-type and mutant NtrC by scanning force microscopy has recently been completed (Wyman *et al.*, 1997).

### Closed complexes of RNAP· $\sigma^{54}$ at the *glnA* promoter

RNAP· $\sigma^{54}$  can bind to the promoter, but it cannot catalyze the melting of the DNA at the promoter on linear templates in the absence of the activator NtrC protein and ATP. This represents an important difference from the RNAP· $\sigma^{70}$  holoenzyme, which does not require an activator for open complex formation at 37°C. Thus, by choosing appropriate reaction conditions, both closed and open promoter complexes of RNAP· $\sigma^{54}$  can be studied separately at this temperature. Figure 4 shows RNAP· $\sigma^{54}$  molecules bound to the *glnA* promoter located about one-fourth of the template length away from the *PstI* end of the template (Figure 1), corresponding to closed promoter complexes. In some of these complexes the RNAP· $\sigma^{54}$  seems to site astride the DNA template (Figure 4a to c), whereas in others (Figure 4d to f) the polymerase appears to be located on the side of the DNA fragment.



**Figure 3.** Binding of NtrC<sup>D54E,S160F</sup> to the DNA template. a to d, The 250 nm × 250 nm SFM images of NtrC<sup>D54E,S160F</sup> bound to one end of the template where the two NtrC binding sites are located (see Figure 1). The different size of the protein complexes indicates the formation of various association states of NtrC<sup>D54E,S160F</sup>.

### Looping between NtrC<sup>D54E,S160F</sup> and RNAP· $\sigma^{54}$

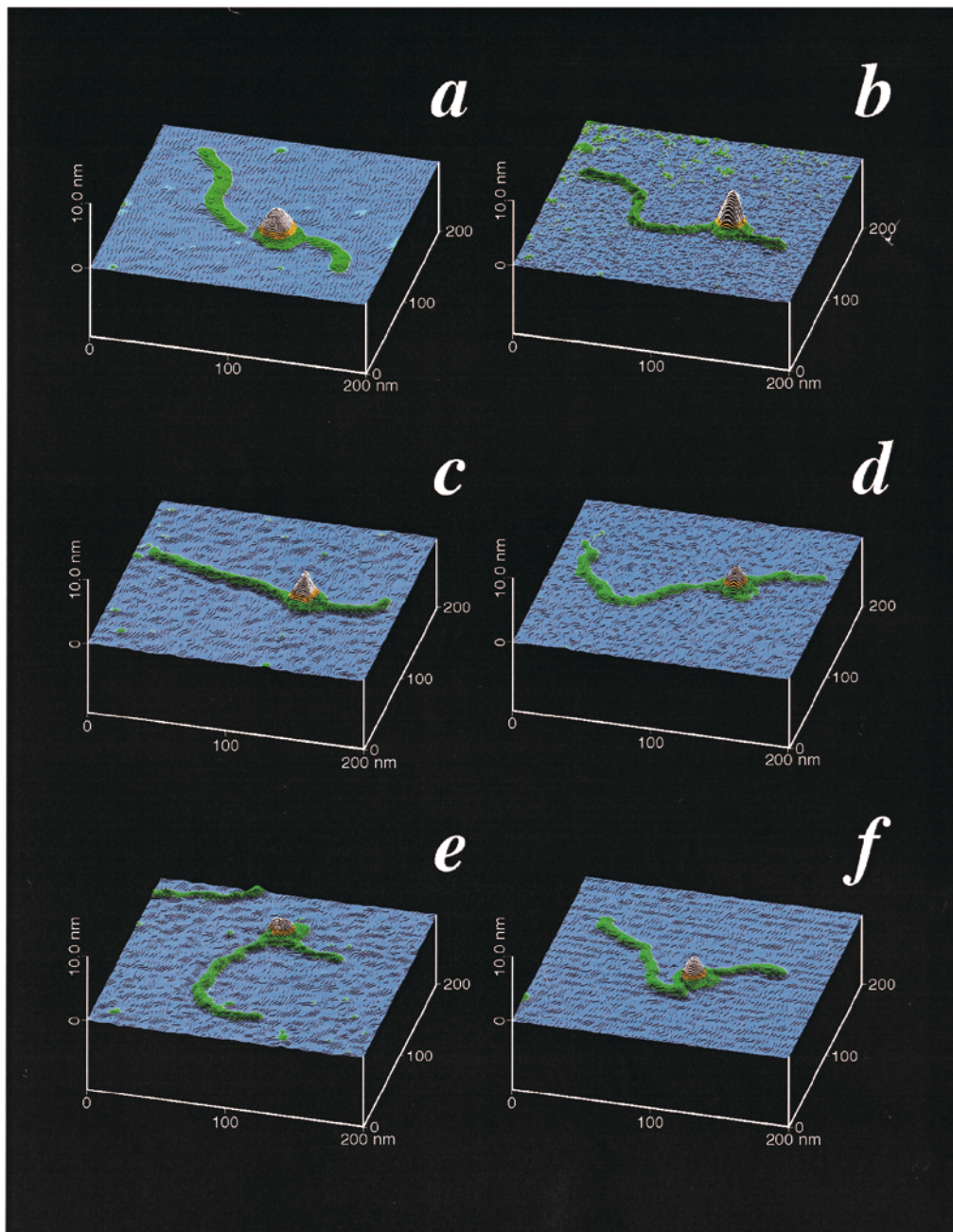
The closed promoter complex of RNAP· $\sigma^{54}$  at the *glnA* promoter can isomerize to an open promoter complex upon addition of NtrC<sup>D54E,S160F</sup> and ATP. This reaction requires protein-protein contacts between NtrC<sup>D54E,S160F</sup> and RNAP· $\sigma^{54}$ , with the intervening DNA being looped out. These looped intermediates were visualized by SFM as shown in Figure 5. An incubation time of five minutes after addition of ATP was found to be optimal for detecting loops. At this incubation time about 15 to 20% of the DNA-fragments that have both protein complexes bound at the expected sites showed looped structures in which NtrC<sup>D54E,S160F</sup> and RNAP· $\sigma^{54}$  appeared to contact one another. In the absence of ATP this number was reduced to 3 to 5%. On a number of images the two different proteins could be clearly distinguished (e.g. Figure 5a, c and f). However, the resolution of the system did not allow an unequivocal identification of the spatial relationship between the two proteins and their respective DNA fragments. The average size of the looped DNA was about 130 nm, a figure

consistent with the positions of the binding sites for NtrC<sup>D54E,S160F</sup> and RNAP· $\sigma^{54}$  on the template.

### Open RNAP· $\sigma^{54}$ complexes with NtrC<sup>D54E,S160F</sup>

The incubation time of the samples was extended to 20 minutes to permit the investigation of the structural changes accompanying the formation of open promoter complexes of RNAP· $\sigma^{54}$  at the *glnA* promoter in the presence of ATP and NtrC<sup>D54E,S160F</sup>. The kinetics of the *in vitro* experiments described above (see Figure 2) suggest that the number of open complexes formed should be highest at this incubation time (data not shown). A set of typical images of molecules that have both protein complexes bound at their respective binding sites is displayed in Figure 6. The NtrC<sup>D54E,S160F</sup> complex in some of these images appeared to be about the same size as RNAP· $\sigma^{54}$  (e.g. compare Figure 6a, b and c), suggesting that in this case the multimeric NtrC<sup>D54E,S160F</sup> complex consisted of four dimers, resulting in a total molecular mass of 440 kDa, which is close to that of RNAP· $\sigma^{54}$ . In other images the two protein complexes were clearly of different size, with both a smaller (e.g.





**Figure 4.** Closed complexes of RNAP· $\sigma^{54}$  at the *glnA* promoter. a to f, The 200 nm  $\times$  200 nm SFM images of the RNAP· $\sigma^{54}$  closed complex at the promoter. According to the position of the *glnA* promoters RNAP· $\sigma^{54}$  binds about one-quarter away from the *Pst*I end of the DNA fragment (Figure 1).

Figure 6e and f) or a larger (Figure 6d) NtrC<sup>D54E,S160F</sup> complex. These images are most consistent with the interpretation that also complexes of NtrC<sup>D54E,S160F</sup> formed that consisted of either two or more than four dimers.

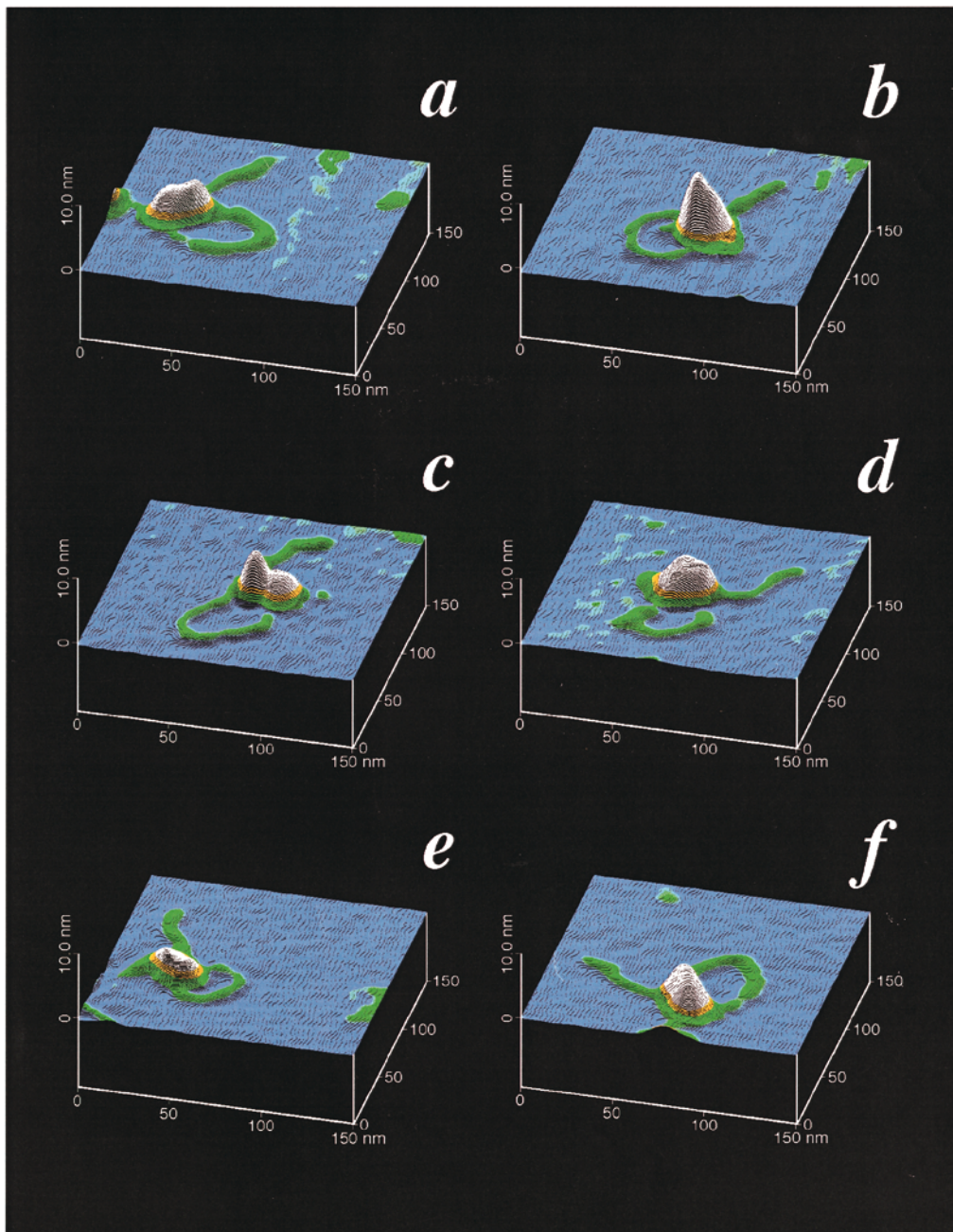
#### Analysis of closed and open RNAP· $\sigma^{54}$ complexes

A structural feature of RNAP· $\sigma^{54}$ -DNA complexes that can be derived from the SFM images is the apparent DNA bending induced by RNA

polymerase at different stages of the transcription process (Rees *et al.*, 1993). In Figure 7a the distribution of bending angles is shown for the closed complexes (compare also Figure 4). The histogram can be fit to a Gaussian distribution yielding an average bending angle of  $49(\pm 24)^\circ$  with an  $r$  value of  $r = 0.97$ . The parameter  $r$  (Pearson's  $r$ ) describes the quality of the fit and contains the sum of the squared differences between actual data points and fit values (chi square) with  $r = 1$  corresponding to a perfect agreement between data and fit.

The distribution of DNA bending angles, measured under conditions that allowed the transition to the open complex (i.e. in the presence of ATP and NtrC<sup>D54E,S160F</sup> with 20 minutes incubation), is presented in Figure 7b). The histogram appears to reflect the bending angle of at least two species. A fit to a single Gaussian distribution yields an average of  $64(\pm 45)^\circ$  with  $r = 0.87$ . The fit is much better if two species are assumed to co-exist in solution. A fit of the data to a sum of two Gaussian distributions centered at  $50(\pm 24)^\circ$  and  $114(\pm 18)^\circ$ , respectively, gave an  $r = 0.95$ . The small

bend angle population is twice as large as that of the higher angle population, judging from the integrated area of the two curves. It is expected that a significant portion of the molecules will not have undergone the transition to the open promoter complex under the experimental conditions used here. The most straightforward interpretation of these data is therefore that the bimodal distribution observed in Figure 7b results from the superposition of a population of closed promoter complexes with an average bending angle of  $50^\circ$  as observed in Figure 7a, and a population of open promoter



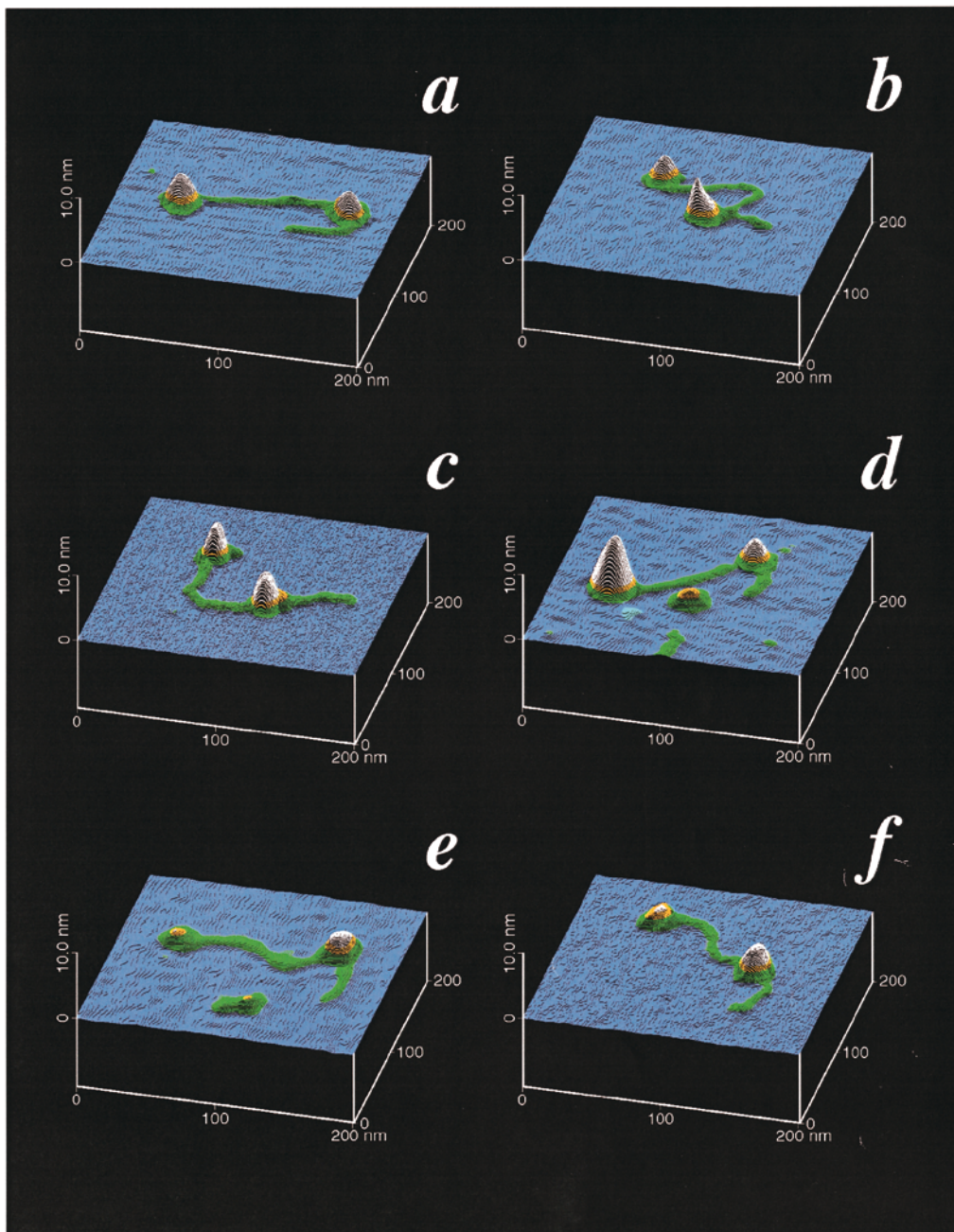
**Figure 5.** Contacts between RNAP· $\sigma^{54}$  and NtrC<sup>D54E,S160F</sup> mediated by DNA-looping. a to f, The  $150\text{ nm} \times 150\text{ nm}$  SFM images of DNA-looping between RNAP· $\sigma^{54}$  and NtrC<sup>D54E,S160F</sup>. The DNA loop had the expected length of about  $130\text{ nm}$  (Figure 1). In some images two distinct protein complexes can be identified (e.g. c and f) whereas in others one large complex appeared (e.g. b and d).



complexes with an average DNA bending angle around  $110^\circ$ .

The *glnA* promoter did not reveal any significant intrinsic DNA curvature, which is shown in the histogram in Figure 7c. A circular mask with the apparent dimensions of the RNA polymerase (20 nm diameter) was placed at the position of the promoter to determine the DNA bending angle at this location in the absence of RNAP· $\sigma^{54}$  (see Materials and Methods). The fit to a Gaussian distribution is excellent ( $r = 1.00$ ). As expected for an unbend DNA fragment it is centered around  $0^\circ$ .

The standard deviation is  $32^\circ$  and the average bending angle is  $25^\circ$ . This  $25^\circ$  bend corresponds to an average end-to-end distance of 19.6 nm and a DNA contour length of 20.2 nm (assuming that the DNA makes a smooth curve within the 20 nm circle). Using equation (9) of Rivetti *et al.* (1996) this corresponds to a persistence length of 54 nm, which is very close to the value of 53 nm determined in the same paper for several linear DNA fragments. Thus, the DNA template at the promoter region does not appear to be different from a random DNA sequence with respect to its flexibility.



**Figure 6.** Open complexes of RNAP· $\sigma^{54}$  at the *glnA* promoter. a to f, The 200 nm  $\times$  200 nm SFM images of complexes formed in the presence of ATP and with 20 minutes incubation time. Only complexes that had both proteins bound and were not looped are presented. Some complexes are relatively strongly bent at the promoter (see a, b and d), whereas in others only a moderate DNA bend is seen (see c and f).

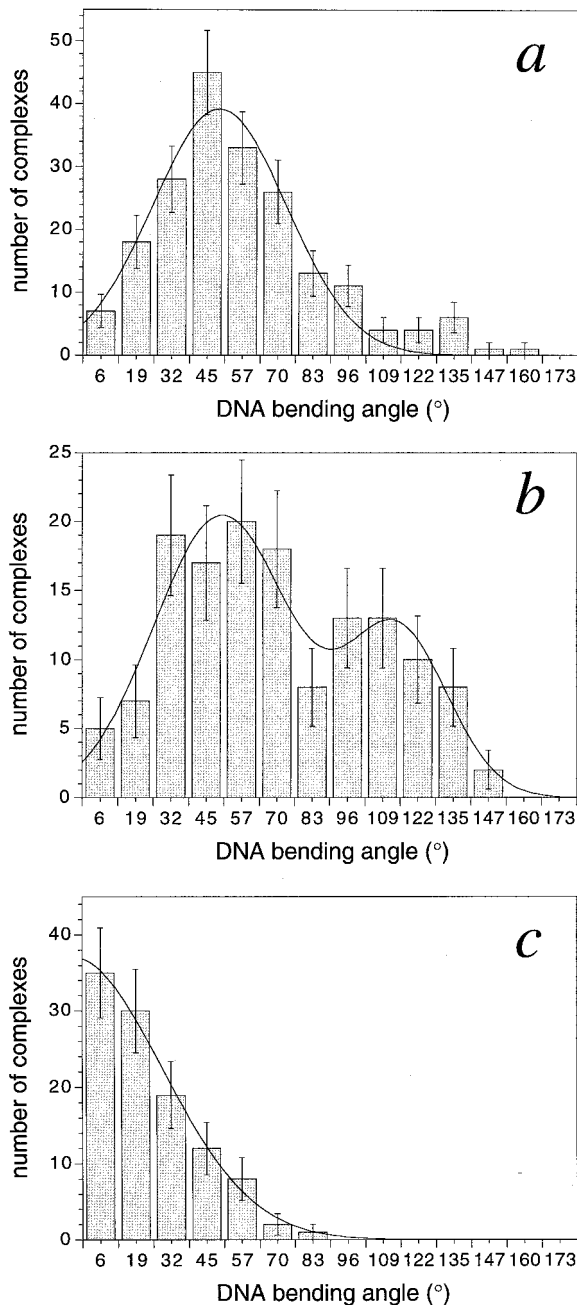
The apparent contour length for the free DNA and the DNA complexed with the various proteins was also measured yielding values of  $254(\pm 4)$  nm (free DNA),  $244(\pm 11)$  nm (NtrC<sup>D54E,S160F</sup> complexes),  $234(\pm 14)$  nm (RNAP· $\sigma^{54}$  closed complexes), and  $217(\pm 20)$  nm (DNA with NtrC<sup>D54E,S160F</sup> and RNAP· $\sigma^{54}$  bound in the presence of ATP to form open complexes). The

expected contour length for the fragment of 726 bp used here is 247 nm for B-DNA (helical rise of 0.34 nm/base-pair), which is in reasonable agreement with the measured value of  $254(\pm 4)$  nm.

## Discussion

It is widely accepted that DNA-looping plays an important role in eukaryotic transcription regulation by mediating interactions between proteins bound at distant sites on the same DNA strand. However, only very few experimental studies directly support this mechanism (see, for example Dunaway & Dröge, 1989; Müller *et al.*, 1989). One reason for the scarcity of experimental studies is the complexity of the eukaryotic transcription system that makes quantitative studies with defined components difficult. A more detailed understanding of this important process requires the development of new methods that allow the direct detection of the conformation changes that occur during the activation process. In this paper, a relatively simple prokaryotic enhancer system consisting of a constitutive mutant of the NtrC activator protein (NtrC<sup>D54E,S160F</sup>), *E. coli* RNA polymerase· $\sigma^{54}$ , and a DNA template with the *glnA* promoter was studied, and transcription activation via DNA-looping was followed by using scanning force microscopy (SFM).

In general, the tethering of two proteins (or protein complexes) on the same piece of DNA can increase the local concentration of one protein in the proximity of the other, and thereby increase the probability of protein-protein contacts (Rippe *et al.*, 1995). The DNA linkage potentiates interactions that would not occur if the proteins were free in solution. Therefore, the efficiency of the transcription activation process is determined, at least to some extent by the probability of contact between the two DNA-bound protein complexes. This probability, in turn, will depend on the DNA conformation. The contact probability may be expressed as the local concentration of NtrC<sup>D54E,S160F</sup> at the promoter due to DNA looping. This parameter can be calculated as described by Rippe *et al.* (1995) for different DNA conformation and is about  $5 \times 10^{-6}$  M (with pJES534 at native superhelicity, with a 460 bp distance between the NtrC binding sites and the promoter),  $1 \times 10^{-7}$  M (with the *EcoRI-PstI* fragment, with a 460 bp distance between the NtrC binding sites and the promoter), and  $1 \times 10^{-8}$  M (with pJES534 linearized with *NcoI*, and a 3000 bp distance between the NtrC binding sites and the promoter) for the three templates used in the *in vitro* transcription assays (Figure 2). The calculated values are in qualitative agreement with the amount of transcript detected (Figure 2, lanes 4, 6 and 8) but quantitative evaluation would require determination of the rate of open complex formation under a variety of conditions. In particular, the fact that the melting of the DNA at the promoter is facilitated in the super-



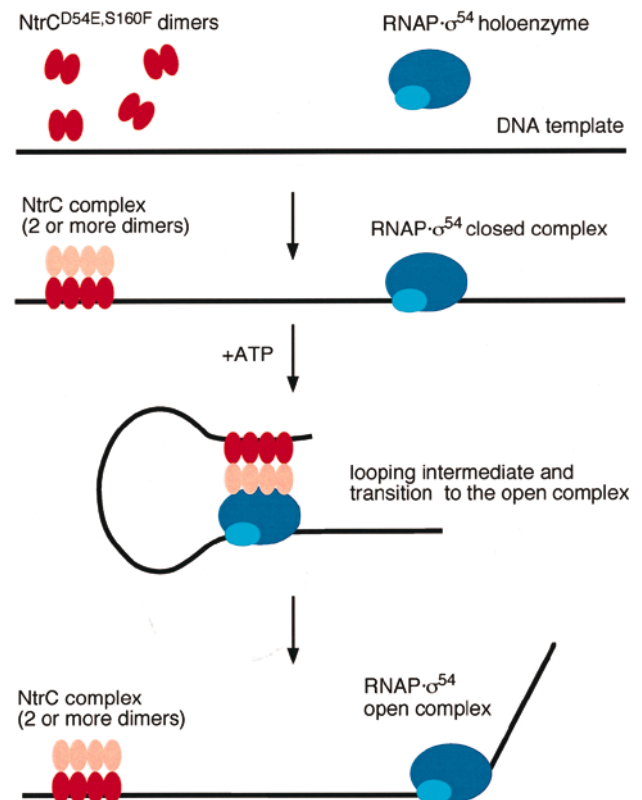
**Figure 7.** Histogram on bending angle of closed/open complexes. (a) Histogram of bending angles for the closed complex of RNAP· $\sigma^{54}$ . (b) Histogram of bending angles measured under conditions where the open complex of RNAP· $\sigma^{54}$  can form (presence of NtrC<sup>D54E,S160F</sup> and ATP, 20 minutes incubation time). (c) Histogram of bending angles at the *glnA* promoter in the absence of RNAP· $\sigma^{54}$ .



helical template has to be taken into account for comparison of superhelical and linear templates. A detailed analysis of the increase of the local protein concentration due to DNA-looping is beyond the scope of the present report, where the focus lies on the visualization of the various species that lead to the initiation of transcription. Nevertheless, it should be noted that also in other studies (e.g. see Carmona & Magasanik, 1996) a strong dependence of the activation rate on the conformation of the DNA template has been observed that at least qualitatively correlates with the expected local concentration of the NtrC complex at the enhancer in the vicinity of the promoter.

A schematic overview of the entire activation process is depicted in Figure 8. The reaction starts with NtrC<sup>D54E,S160D</sup> binding at the tandem enhancer site and RNAP· $\sigma^{54}$  binding at the *glnA* promoter to form the closed complex. The closed complex of RNAP· $\sigma^{54}$  is apparently too unstable to be visualized by conventional electron microscopy (Su *et al.*, 1990), yet it has been successfully imaged by SFM (Figure 4). The images of the closed complexes suggest that RNAP· $\sigma^{54}$  forms an asymmetric structure in which the DNA template is bound to one side of the holoenzyme (see Results). Such a conformation has also been observed for RNAP· $\sigma^{70}$  by SFM (Rees *et al.*, 1993). As described in Materials and Methods, the SFM samples were prepared under conditions very similar to those used for the *in vitro* transcription assays, without any further processing to enhance contrast. This minimal processing of the materials is likely to be important for preserving the relatively weak DNA-protein and protein-protein interactions during the deposition process. This situation is relevant for the characterization of the different oligomeric states of NtrC as well. The association of NtrC dimers is important for the formation of an active NtrC complex, but the resulting complex is too unstable to be studied by gel electrophoresis (Porter *et al.*, 1993). With wild-type NtrC, the oligomerization is stimulated by phosphorylation (Porter *et al.*, 1993; Weiss *et al.*, 1992b). In this study, different oligomers of NtrC<sup>D54E,S160F</sup> formed at the two binding sites of the DNA template were detected by SFM, revealing higher order complexes of NtrC<sup>D54E,S160F</sup> dimers (Figures 3 and 6), that have also been observed by electron microscopy (Porter *et al.*, 1993; Révet *et al.*, 1995; Su *et al.*, 1990). The use of SFM offers additional information on the dimensions of the sample in the Z-direction, which is important for determining how many NtrC dimers are present in a given complex. A comprehensive and quantitative analysis of this issue by scanning force microscopy is presented by Wyman *et al.* (1997). The complex formed by NtrC<sup>D54E,S160F</sup> in Figure 8 is shown to be a tetramer of dimers, but other association states can also be detected in the SFM images, as described above.

A looped complex that can form in the presence of NtrC<sup>D54E,S160F</sup>, RNAP· $\sigma^{54}$  and ATP, is the next intermediate in the reaction pathway and is de-



**Figure 8.** Scheme of the activation process. The species that can be observed by scanning force microscopy are shown. The template has two binding sites for NtrC. Additional NtrC<sup>D54E,S160F</sup> dimers (drawn in pink) can bind by protein-protein interactions and a tetramer of NtrC<sup>D54E,S160F</sup> dimers is shown. Other association states have also been detected (see Figures 3 and Figure 6). The closed complex of RNAP· $\sigma^{54}$  at the *glnA* promoter also forms in the absence of NtrC<sup>D54E,S160F</sup> and is shown in Figure 4. In the presence of ATP the activation process proceeds *via* the formation of a looped intermediate (Figure 5). In this complex NtrC<sup>D54E,S160F</sup> catalyzes the transition to the open complex of RNAP· $\sigma^{54}$  (Figure 6) upon hydrolysis of ATP. As deduced from the measurements made here and the increases of the footprint (Popham *et al.*, 1989) this isomerization is accompanied by a change of the DNA bending angle at the promoter, as indicated in the Figure.

icted in Figure 8. The loops can be detected by scanning force microscopy, as shown in Figure 5. These results confirm previous work on wild-type NtrC in which images of looped complexes were obtained by conventional electron microscopy (Su *et al.*, 1990). Another important study (Wedel *et al.*, 1990) has shown that activation by NtrC does not require a continuous DNA path between the NtrC binding site and the promoter. Taken together these results provide strong evidence that transcriptional activation in the NtrC/RNAP· $\sigma^{54}$  system involves direct protein-protein contacts between activator and polymerase that are mediated by DNA-looping, in contrast to many eukaryotic

systems where this mechanism has been postulated, but not unequivocally demonstrated. To obtain a more detailed characterization of the loop conformation it would be important to follow the path of the DNA, to determine whether there is a cross-over of the two DNA strands or a strong DNA bend at the promoter. This is not possible on the images obtained so far, and would require other templates with labels introduced at specific positions along the DNA, and identification of NtrC and RNAP· $\sigma^{54}$  in the complex. For the latter purpose, it might be advantageous to use electron microscopy, since RNAP· $\sigma^{54}$  can be distinguished from NtrC by its darker color after tungsten shadowing (Su *et al.*, 1990).

The ability to study both the closed and open complexes of RNAP· $\sigma^{54}$  by SFM made it possible to analyze conformational differences between the two forms of the RNA polymerase holoenzyme. Measurements of the apparent DNA bending angles of RNAP· $\sigma^{54}$  at the *glnA* promoter revealed that this parameter was different in the closed and in the open promoter complex and can therefore be used as an indicator of the RNAP· $\sigma^{54}$  conformation. Under conditions where only the closed complex can be formed, a single distribution with an average bending angle of  $50^\circ$  was observed (Figure 7a). The distribution of bending angles measured in the presence of NtrC<sup>D54E,S160F</sup> and ATP, which catalyzes open complex formation, appears to be the sum of two distributions: one with a peak at around  $50^\circ$  that is likely to correspond to closed promoter complexes and the other that is centered around  $110^\circ$ , which probably represents the open promoter complexes. This suggests that the transition from closed to open promoter complexes in RNAP· $\sigma^{54}$  is accompanied by a large increase in DNA bending at the promoter that is likely to reflect large changes in the spatial relationships between the DNA and the polymerase.

To what extent does the DNA bending angles measured here by SFM represent the "true" DNA bending of promoter bound RNAP· $\sigma^{54}$  free in solution? This question has been discussed in the context of other SFM studies (Erie *et al.*, 1994; Rees *et al.*, 1993; Rivetti *et al.*, 1996; C. Walker & C. Bustamante, unpublished results). In particular, the latter two papers provide a detailed account of the relation between DNA bending in solution to that measured by SFM after deposition on the surface. From this work and the comparison of cases in which a DNA bending angle has been determined both by SFM and by another method (e.g. gel electrophoretic analysis or X-ray crystallography), it appears that no systematic error is introduced in the mean DNA bending angle that exists in solution under the conditions of deposition used here. In addition it should be noted that even if the absolute values of the mean DNA bending angles would be affected by the deposition, it is unlikely that these same effects could result in the different

distributions observed in this study for closed and open promoter complexes.

The increase of the DNA bending angle in open complexes is in good agreement with the observed change in the DNA footprint. In the closed complex the DNA at the *glnA* promoter is protected in the template region between  $-30$  and the transcription start site at position  $+1$ , whereas in the open complex the protected region is extended by 20 bp and reaches from template position  $-30$  to about  $+20$  (Popham *et al.*, 1989). This change in the footprint indicates that the RNA polymerase holoenzyme makes additional contacts with the DNA in the open complex that manifest themselves as an increase of the DNA bending angle as the DNA wraps around the protein. The significant decrease of the apparent DNA contour length observed here for the closed RNAP· $\sigma^{54}$  complexes, which was even more pronounced under conditions where the transition to the open complex can occur (see Results), is consistent with this model.

Wrapping of the DNA around *E. coli* RNAP· $\sigma^{70}$  holoenzyme has also been suggested on the basis of topological studies (Amouyal & Buc, 1987), and it is expected from purely geometric considerations: the dimensions of RNAP· $\sigma^{70}$  have been determined by electron diffraction of two-dimensional crystals and it has been concluded that bending of the DNA would be necessary for RNA polymerase to interact with over 50 to 60 bp in the open promoter complex, which corresponds to at least 170 Å of B-form DNA (Darst *et al.*, 1989).

An increase in the size of the footprint similar to that observed with RNAP· $\sigma^{54}$ , has also been reported for RNAP· $\sigma^{70}$  (Schickor *et al.*, 1990, and references therein), showing protection from about position  $-53$  to  $-4$  in the closed complex and  $-53$  to  $+20$  in the open complex. However, it should be noted that in these experiments the analysis of the closed complex requires a temperature of 0 to  $4^\circ\text{C}$  and the transition to the open complex is induced by incubation at a temperature of  $37^\circ\text{C}$ . For RNAP· $\sigma^{54}$  the analysis can be done at the same temperature because the formation of the open complex is induced by incubation with NtrC and ATP.

Open RNAP· $\sigma^{70}$  complexes formed on the  $\lambda$  P<sub>L</sub> promoter display a multimodal distribution of bending angles with three peaks around 0 to  $10^\circ$ , 30 to  $70^\circ$  and 80 to  $110^\circ$  (see Figure 4a of Rees *et al.*, 1993). This distribution is likely to represent a mixture of closed and open complexes and is very similar to that for RNAP· $\sigma^{54}$  shown here in Figure 7b with the exception of the peak at 0 to  $10^\circ$ , which is not observed with RNAP· $\sigma^{54}$ . Thus, it is tempting to speculate that the transition from the closed to the open complexes for both RNAP· $\sigma^{54}$  and RNAP· $\sigma^{70}$  is accompanied by an increase in the DNA bending angle, reflecting an extension of the area of the DNA template that is in contact with the polymerase.

## Concluding remarks

The work presented here, together with other SFM studies (e.g. see Guthold *et al.*, 1994; Hansma *et al.*, 1993; Kasas *et al.*, 1997; Rees *et al.*, 1993; Wyman *et al.*, 1995), demonstrates that scanning force microscopy is a valuable addition to the available repertoire of techniques that can be applied to elucidate the mechanism of the transcription activation process. SFM offers relative ease of operation and sample preparation for the visualization of DNA and DNA-protein complexes at nanometer resolution under conditions that preserve important features of the native conformation. In addition SFM can be applied to studies of the type presented here, but in physiological buffers and including the possibility of changing solution conditions. It is anticipated that this capability of the method will prove useful to biochemists in a variety of mechanistic studies of biological processes.

## Materials and Methods

### Plasmid construction and DNA purification

The protein expression vectors pS54-2 and pNTRC-2 were created to permit over-expression of His-tagged  $\sigma^{54}$  and NtrC<sup>D54E,S160F</sup>. The  $\sigma^{54}$  gene was PCR amplified from pTH7 (obtained from Jay Gralla and originally constructed as described by Hunt & Magasanik, 1985) with the primers ATT CTG **CAT ATG AAG CAA GGT TTG CAA** (*NdeI* site in bold) and CGT AAA CAA CTC GTT TGA **CTC GAG CGA TCC** (*XhoI* site in bold) and Pfu polymerase from Stratagene under standard PCR conditions. The primers introduced the indicated *NdeI* and *XhoI* sites. The resulting PCR product was digested with *NdeI* and *XhoI* and was cloned into the same sites of pET15b to make pS54-1. In pS54-1 the *Sall-Nsil* fragment in the  $\sigma^{54}$  gene was replaced with the same fragment from pTH7 to make plasmid pS54-2. This step was added to avoid the introduction of mutations by the PCR amplification in this region. The integrity of the flanking regions of the  $\sigma^{54}$  gene was then checked by DNA sequencing.

The plasmid pJES457, carrying the NtrC<sup>D54E,S160F</sup> gene from *Salmonella* (obtained from Sydney Kustu), was cut with *NdeI*-*BamHI*, and the resulting fragment was ligated into the same sites in pET15b to construct plasmid pNTRC-2.

The DNA fragment used for the protein binding and transcription reactions was the 726 bp linear *EcoRI-PstI* from pJES534 (obtained from Sydney Kustu). The fragment was purified from SeaKem GTG agarose using the Qiagen QIAquick gel extraction kit. The eluted DNA fraction was further purified by three times phenol, three times ether extraction and ethanol precipitation and was then resuspended in TE buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA). DNA fragment concentrations were determined by absorbance measurements using an extinction coefficient of 6500 M<sup>-1</sup> cm<sup>-1</sup> per nucleotide at 260 nm.

### Protein purification

The  $\sigma^{54}$  and the NtrC<sup>D54E,S160F</sup> proteins were expressed with a N-terminal His-tag from pS54-2 and pNTRC-2, re-

spectively, in a T7 RNA polymerase expression system (Studier, 1990). Competent *E. coli* BL21 (DE3) carrying the pLysS plasmid were transformed with pS54-2 or pNTRC-2. A single colony from the transformed cells was used to inoculate 10 ml LBC medium (LB supplemented with 200  $\mu$ g/ml carbenicillin and 34  $\mu$ g/ml chloramphenicol). This culture was grown at 37°C until the  $A_{600}$  (absorbance at 600 nm) was 0.6 to 1.0, and then stored overnight at 4°C. The next day the cells were spun down and washed once with fresh medium and then resuspended in 1 ml of LBC medium and used to inoculate 250 ml of medium with this culture. The cells were grown at 25°C to an  $A_{600}$  of 0.6 to 1. Protein expression was induced by adding 0.1 M IPTG to a final concentration of 1 mM. The cells were harvested by centrifugation after three hours of growth and the pellet was stored at -20°C prior to further purification.

For protein purification the frozen cell pellet was dissolved in 40 ml of binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl (pH 7.9), 10 mM  $\beta$ -mercaptoethanol), supplemented with Nonidet-P40 to a final concentration of 0.1% and 1 mM PMSF. The cells were lysed by sonication on ice, and then centrifugated at 40,000 g for 20 minutes. The supernatant was filtered through a 0.45  $\mu$ m filter and loaded onto a HR10-10 column packed with Ni NTA-agarose (Qiagen) that had been equilibrated with binding buffer at a flow rate of 1 ml/min. The column was washed with binding buffer until the measured  $A_{280}$  was stable, and then washed with washing buffer (60 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl (pH 7.9), 10 mM  $\beta$ -mercaptoethanol) and eluted with elution buffer (500 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl (pH 7.9), 10 mM  $\beta$ -mercaptoethanol). The eluted peak fractions were collected, EDTA was added to a concentration of 5 mM, and the proteins were dialyzed against buffer A (20 mM Tris-HCl (pH 7.9), 50 mM KCl, 0.1 mM EDTA, 1 mM DTT, 5% (v/v) glycerol). The  $\sigma^{54}$  preparations were purified on a MonoQ anion exchange column with a flow rate of 0.5 ml/min and a gradient from 0.05 M to 0.75 M KCl in 150 ml. A flow rate of 0.5 ml/min and a gradient from 0.05 M to 0.5 M KCl in 150 ml was used for the NtrC<sup>D54E,S160F</sup> preparations. The peak fraction of  $\sigma^{54}$  (eluted at ~0.45 M KCl) and of NtrC<sup>D54E,S160F</sup> (eluted at ~0.20 M KCl) were pooled and dialyzed against 40 mM Tris-HCl (pH 7.9), 100 mM KCl, 0.2 mM EDTA, 2 mM DTT. Glycerol was added to a final concentration of 50% (v/v), and the samples were stored at -20°C.

A partially purified mixture of *E. coli* RNA polymerase core and holoenzyme was kindly provided by Kevin Wilson. Core and holoenzyme were separated on a MonoQ column as described (Hager & Burgess, 1990). The *E. coli* RNA polymerase  $\sigma^{70}$ -holoenzyme and core RNA polymerase preparations were stored at -20°C in 10 mM Tris-HCl (pH 7.9), 0.1 M NaCl, 0.1 mM EDTA, 0.1 mM DTT, 50% glycerol. *E. coli* RNA  $\sigma^{54}$ -holoenzyme was prepared by mixing core RNA polymerase and  $\sigma^{54}$  at a ratio of 1 to 1.5 and was stored at -20°C in 10 mM Tris-HCl (pH 7.9), 0.1 M NaCl, 0.1 mM EDTA, 0.1 mM DTT in 50% glycerol.

Protein concentrations were determined by absorbance measurements using extinction coefficients at 280 nm of 42,900 M<sup>-1</sup> cm<sup>-1</sup> ( $\sigma^{54}$ ), 46,400 M<sup>-1</sup> cm<sup>-1</sup> (NtrC<sup>D54E,S160F</sup>), and 198,500 M<sup>-1</sup> cm<sup>-1</sup> (core RNAP) as calculated from the amino acid composition (Gill & von Hippel, 1989).



### Transcription reactions

Single round transcription assays were performed by a procedure similar to that described by Klose *et al.* (1993). RNAP· $\sigma^{54}$  (or RNAP· $\sigma^{70}$  in lanes 1 and 2) at a concentration of 60 nM, and NtrC<sup>D54E,S160F</sup> at 240 nM (if present), were incubated with 6 nM of the various DNA templates in HAM buffer (40 mM Hepes/KOH (pH 8.0), 10 mM magnesium acetate, 100 mM potassium acetate, and 0.1 mM DTT) for ten minutes at 37°C to allow protein binding. Open complex formation was initiated by adding ATP to 5 mM and the samples were incubated for another ten minutes. Then a mixture of GTP (to 1 mM), CTP[ $\alpha$ -<sup>32</sup>P]CTP (to 25  $\mu$ M) and heparin (to 100  $\mu$ g/ml) in HAM buffer was added to permit for a single transcription cycle. After ten minutes the reaction was stopped by ethanol precipitation and the radioactive transcripts were analyzed on a 8% (w/v) sequencing gel.

### Scanning force microscopy

DNA-protein complexes were formed by incubating 20 nM of the 726 bp *EcoRI-PstI* fragment from pJES534 with 60 nM RNAP· $\sigma^{54}$  and/or 500 nM NtrC<sup>D54E,S160</sup> in HAM buffer (see above) for ten minutes at room temperature. Open complex formation was initiated by addition of a 100 mM ATP solution to a concentration of 5 mM and incubation for five minutes (for detection of loops) or 20 minutes (for detection of RNAP· $\sigma^{54}$  open complexes) at 37°C. ATP was omitted for imaging NtrC<sup>D54E,S160</sup> complexes (Figure 3) or the closed complex of RNAP· $\sigma^{54}$  (Figure 4). After incubation of the reaction for the given times, 4  $\mu$ l of the reaction mix were diluted into 36  $\mu$ l of SFM buffer (20 mM Hepes/KOH (pH 8.0), 10 mM magnesium acetate, 30 mM potassium acetate) and 10  $\mu$ l of this solution were immediately deposited onto a piece of freshly cleaved mica. The mica disc was washed right away by dropping distilled water onto the surface and then drying the sample in a stream of N<sub>2</sub>. The images were obtained in air at ambient humidity with a Nanoscope III (Digital Instruments, Santa Barbara, CA) in the tapping mode, using silicon tips from Digital Instruments, Santa Barbara, CA.

### Analysis of DNA bending angles and measurements of DNA contour length

The DNA bending angles of RNAP· $\sigma^{54}$  at the promoter were measured with the installed Nanoscope Software by drawing lines through the DNA axes on both sides of the polymerase and measuring the angle  $\alpha$  at their intersection (Rees *et al.*, 1993). The DNA bending angle  $\theta$  is then defined as  $\theta = 180^\circ - \alpha$ . Only molecules that had the polymerase located at the approximate position of the *glnA* promoter were evaluated. To determine the bending angle of open complexes only those molecules were selected that had both NtrC<sup>D54E,S160F</sup> bound to the end of the DNA fragment and the RNAP· $\sigma^{54}$  at the promoter. Looped complexes were not included. In the analysis 197 closed complexes were evaluated, 140 complexes that were formed under conditions allowing the transition to the open complex (presence of ATP, NtrC<sup>D54E,S160F</sup>, 20 minutes incubation time), and 107 molecules to determine the intrinsic DNA bending angle at the *glnA* promoter. For the latter distribution, DNA fragments with NtrC<sup>D54E,S160F</sup> bound were used to locate the *PstI* end of the strand with the promoter. Then a circular mask with the apparent dimensions of the RNA polymerase (20 nm diameter) was placed at the position of

the promoter and the DNA bending angle was determined. At least three independent experiments were analyzed for the different complexes studied. No dependence on deposition was detected within the errors of the experiment. The distributions of DNA bending angles obtained were categorized into a number of bins that approximated the square root of the number of samples over the range of values observed. The program Kaleidagraph (Synergy Software, PA) was used to fit the resulting histograms to one Gaussian or to the sum of two Gaussian distributions. The error bars given for each bin correspond to the square root of the number of samples within each bin.

The same complexes for which DNA bend angles were determined were also used for measurements of the DNA contour length. In addition 110 free DNA molecules were also analyzed. The contour length was determined in NIH image v. 1.58 (National Institutes of Health, Bethesda, MD). At the regions where proteins were bound a curve was drawn through the center of the protein from the DNA entry and exit points. With the large NtrC complexes the end of the DNA was sometimes not visible. In these cases the curve from the DNA entry point through the center of the protein was continued as a straight line to the end of the protein complex.

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