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Action of prokaryotic enhancer over a distance does not require continued presence of promoter-bound σ^{54} subunit

Vladimir Bondarenko, Ye Liu, Alexander Ninfa[1] and Vasily M. Studitsky*

Department of Biochemistry and Molecular Biology, Wayne State University School of Medicine, 540 East Canfield Avenue, Room 5123, Detroit, MI 48201, USA and [1]Department of Biological Chemistry, University of Michigan Medical School, 1301 Catherine Road, Ann Arbor, MI 48109-0606, USA

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ABSTRACT

The mechanism by which an enhancer activates transcription over large distances has been investigated. Activation of the *glnAp2* promoter by the NtrC-dependent enhancer in *Escherichia coli* was analyzed using a purified system supporting multiple-round transcription *in vitro*. Our results suggest that the enhancer–promoter interaction and the initiation complex must be formed *de novo* during every round of transcription. No protein remained bound to the promoter after RNA polymerase escaped into elongation. Furthermore, the rate of initiation during the first and subsequent rounds of transcription were very similar, suggesting that there was no functional 'memory' facilitating multiple rounds of transcription. These studies exclude the hypothesis that enhancer action during multiple-round transcription involves the memory of the initial activation event.

INTRODUCTION

Transcriptional enhancers are relatively short (30–200 bp) DNA sequences usually composed of several binding sites for activator protein(s). The landmark of enhancers is their ability to activate genes over a considerable distance (up to 60 kb in Eukaryota and up to 15 kb in Prokaryota) (reviewed in 1,2). Gene regulation over such large distances is widespread in higher Eukaryota. In Prokaryota, enhancer-dependent gene regulation is less common; only a small number of promoters used by σ^{54}-containing RNA polymerase are known to be regulated by enhancers (3–5).

The mechanism of enhancer action over a large distance is unknown. The 'recruitment' mechanism proposed for gene activation involves the recruitment of a promoter-binding protein by an enhancer-bound protein through protein–protein interaction (reviewed in 6). However, it explains the action of activators satisfactorily only over enhancer–promoter distances up to 1 kb. Therefore, it is unclear how efficient communication between enhancer and promoter can occur over distances >2 kb (reviewed in 7). Several models have been proposed to explain the mechanism of enhancer action. One class of models suggests that initial communication of an enhancer with a promoter leads to formation of a stable DNA–protein complex in the vicinity of the promoter. This stable complex may facilitate subsequent rounds of transcription serving as a 'memory' of initial enhancer–promoter contact (reviewed in 1). Conceivably, 'memory' may also be due to alteration of the template. In either case, 'memory' is expected to result in a vast difference in the rates of the first round of transcription (which by definition occurs in the absence of memory) and subsequent rounds of transcription.

Alternatively, the average distance between promoter and enhancer could be considerably decreased if intervening DNA is supercoiled or bent (reviewed in 7). In this case, formation of a stable DNA–protein complex at the promoter (or any other type of memory) may not be required because DNA supercoiling or bending bring the enhancer into close proximity to the promoter and thus allow more efficient recruitment of the transcriptional machinery. In this case, the rate of initiation of the first round of transcription and subsequent rounds of transcription from the promoter should be similar.

The NtrC (NRI)-dependent, σ^{54}-dependent transcriptional enhancer participates in the regulation of genes involved in metabolism of nitrogen in *Escherichia coli* (see Fig. 5) (reviewed in 8). The mechanism of action of the NtrC-dependent enhancer has been intensely studied using the *glnAp2* promoter as a model. The enhancer is localized ~110 bp upstream of the *glnAp2* promoter but strongly activates transcription when positioned up to at least 15 kb away *in vivo* (9) and up to at least 0.9 kb *in vitro* (10); it functions both upstream and downstream from the promoter. NtrC is an activator that binds to the enhancer, and, when phosphorylated by NtrB (NRII) protein kinase, forms a higher order homooligomer and is capable of activating the transcription of the *glnAp2* gene (11–14). Phosphorylation of NtrC also activates its ATPase activity, which is required to stimulate conversion of the closed promoter–polymerase complex (RP_c) to the transcriptionally active open complex (RP_o), in which the strands of the template are melted near the site of initiation (11–14). Active enhancer-bound NtrC interacts with the $E\sigma^{54}$ RNA polymerase holoenzyme bound as the closed initiation complex (RP_c) at the promoter (15–17). During enhancer–promoter interaction,

*To whom correspondence should be addressed. Tel: +1 313 993 7818; Fax: +1 313 577 2765; Email: vstudit@med.wayne.edu

intervening DNA is looped out (18,19). Interaction of the NRI with the σ^{54} subunit of the holoenzyme drives the transition from RP_c into RP_o (20–22).

In this work, a multiple-round *in vitro* transcription assay was employed to investigate the mechanism of NtrC-dependent enhancer action over a large distance. Previously, we have found that DNA supercoiling greatly facilitates prokaryotic enhancer action over a large distance (2.5 kb) but DNA supercoiling is not essential for action over a short distance (0.11 kb) (23). The experiments described here excluded the possibility that the initial activation event provided 'memory' at the promoter facilitating multiple rounds of transcription, and, in contrast, suggested that multiple rounds of transcription occur by the same activation mechanism as the initial round. Possible mechanisms of enhancer action are discussed.

MATERIALS AND METHODS

Purified proteins

All proteins and protein complexes were purified according to protocols previously described for core RNA polymerase (24), σ^{54} (24), NtrC (25) and NtrB (26). Isolated proteins have been analyzed in 10% SDS–PAGE and their purity was over 95% according to Coomassie and silver staining (see Fig. 3A).

DNA templates

For detailed description of all plasmid templates used in the experiments see Figure 1. Supercoiled DNA templates were purified using QIAGEN plasmid purification kit. Plasmids pTH8, pLR100 and pAN6 containing *glnAp2* promoter were previously described by Ninfa *et al.* (10).

In vitro transcription

In vitro transcription was optimized for maximal utilization of promoter in initiation and elongation using supercoiled template pTH8 plasmid as a template. Purified proteins NtrC, NtrB, σ^{54} and core RNA polymerase used in this system were analyzed in a single-round transcription assay on supercoiled template. Exclusion of any of the protein components from the transcription assay abolished transcription from *glnAp2* promoter. The buffer for the transcription assay contained 50 mM Tris-OAc (pH 8.0), 100 mM KOAc, 8 mM $Mg(OAc)_2$, 27 mM NH_4OAc, 0.7% PEG (8000) and 0.2 mM DTT. The transcription reactions were conducted in 50 µl aliquots and the template DNA was present at 2.8 nM. The final (saturating) concentrations of protein components were 500 nM core RNA polymerase, 1000 nM σ^{54}, 120 nM NtrC and 400 nM NtrB. The reaction mix was incubated for 15 min at 37°C to form RP_c. ATP was added to the reaction to 0.5 mM (single-round) or 2 mM (multiple-round) final concentration and reaction mixture was incubated at 37°C for 15 min (or for variable time when the time-course of single-round transcription was analyzed) to form RP_o. Then, NTPs (final concentration 80 µM), 2.5 µCi [α-^{32}P]UTP, RNase inhibitor (final concentration 0.2 U/µl) and heparin (final concentration 80 µg/ml, single-round transcription only) were added to the reaction to start transcription. The mixture was incubated at 37°C for 15 min (or for different times when time-course of multiple-round transcription was analyzed) and then, 50 µl of stop solution (200 µg/ml sheared DNA, 40 mM EDTA) was added to terminate the reaction. End-labeled 227 bp DNA fragments (1–2 µl) were added to the mixture as a loading control. The samples were extracted with 100 µl of phenol–chloroform (1:1), precipitated with ethanol, washed with 70% ethanol and dissolved in 100% formamide. The samples were separated in 8% denaturing urea-containing PAGE, dried and analyzed using a PhosphorImager.

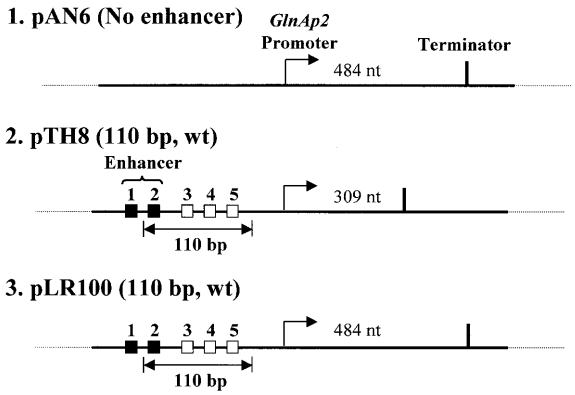

Figure 1. Templates for analysis of the mechanism of *glnAp2* promoter activation by NtrC-dependent enhancer. Plasmid templates having different enhancer–promoter spacing (constructs 2 and 3) and having no enhancer (construct 1). Strong and weak NtrC-binding sites are indicated by closed and open squares, respectively. Under our experimental conditions only the strong sites are occupied and contribute to the enhancer activity (10). The pTH8 and pLR100 plasmids (3.6 and 3.3 kb in size, respectively) have 110 bp wild-type (wt) enhancer–promoter spacing. The transcripts were terminated at the T7 terminator positioned over different distances downstream of the promoter. The lengths of the transcripts are indicated.

DNase I and $KMnO_4$ footprinting

The footprinting was conducted under conditions used in the transcription assays [see above and Sasse-Dwight and Gralla (27)]. After formation of RP_c, ATP was added to the reaction (final concentration 0.5 mM) and samples were incubated at 37°C for 15 min to form RP_o. GTP and CTP were added to the reaction (final concentration 0.05 mM) and incubation continued at 37°C for 10 min to form an elongation complex (RP_{el}). Measurements of the length of the transcript formed in the –U reaction suggest that, consistent with the footprinting data (see Fig. 2), RP_{el} terminates at position +32 (data not shown). The final volumes of the samples were 20 µl each. Before DNase I digestion, the enzyme preparation was calibrated before the experiment to achieve digestion of only 30–40% of protein-free DNA to guarantee primarily single-hit digestion of the samples. After formation of RP_c, RP_o and RP_{el}, 2 µl of DNase I (0.15 U/µl; Sigma) was added and samples were incubated at 37°C for 30 s. The reaction was terminated with 2 µl of 0.5 M EDTA. $KMnO_4$ footprinting was conducted by adding 2 µl of 80 mM $KMnO_4$ and incubation at 37°C for 1 min. The reaction was terminated with 2 µl of 14.7 M 2-mercaptoethanol. After treatment with footprinting reagents, 20 µl of phenol–chloroform (1:1) was added. The samples were heated at 90°C for 4 min, cooled on ice and centrifuged at 14 000 *g* for 2 min. The upper aqueous layer was desalted on a MiniSpin G-50 column equilibrated with 10 mM Tris–HCl, 0.1 mM EDTA buffer. The modified templates were then subjected to primer extension using Klenow fragment (Gibco BRL) and the following ^{32}P-labeled

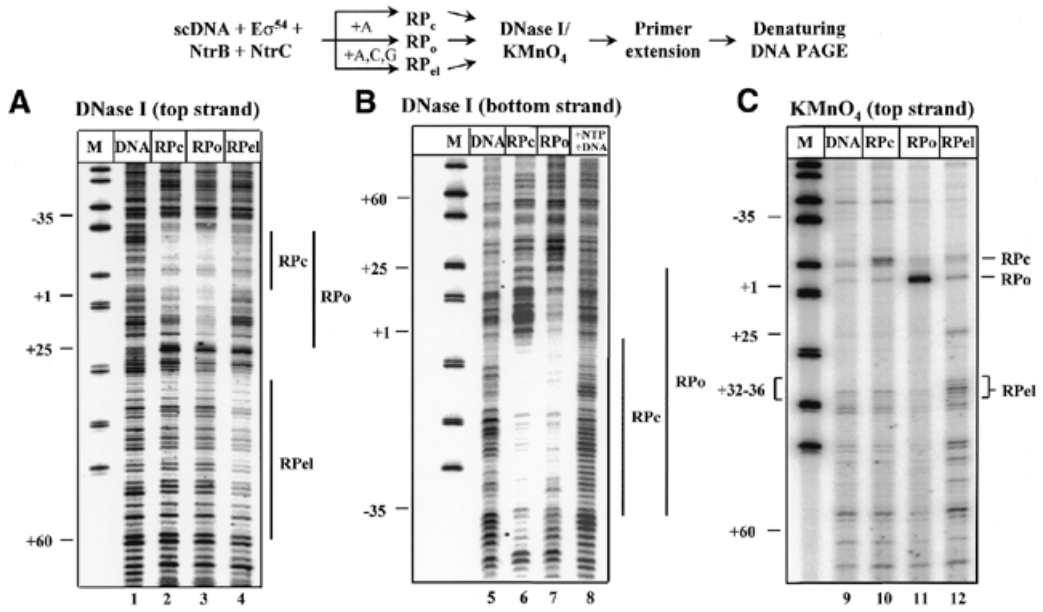

Figure 2. Escape of the RNA polymerase from the *glnAp2* promoter is accompanied by disappearance of $KMnO_4$ and DNase I footprints characteristic for RP_o complex. The experimental strategy for obtaining the RP_c, RP_o and RP_{el} complexes is outlined at the top. In all experiments supercoiled pTH8 plasmid (110 bp enhancer–promoter spacing) was used. Reaction mixtures were incubated in the presence of ATP (+A; RP_o) or partial mixture of nucleotides (+A, C, G; RP_{el}), or in the absence of nucleotides (RP_c). In lane 8, all NTPs were added to RP_o in the presence of a large excess of competitor DNA (pLR90 plasmid, 100 nM). All experiments were performed as described by Tintut *et al.* (29). Protein-free DNA (lanes labeled DNA) was used as a negative control. After formation of the complexes and their incubation in the presence of DNase I (**A** and **B**) or $KMnO_4$ (**C**) the samples were analyzed by single-round primer extension using ^{32}P-labeled primers corresponding to the indicated DNA strand, and resolved in a denaturing PAGE. Vertical bars mark DNase I footprints characteristic for the corresponding complexes.

primers (28,29): 5′-CGTATGGGCTAAAGAATCCCCATT-GACTTAGG (top DNA strand); 5′-TTCACATCGTGGTGC-AGCCC (bottom DNA strand). The products of the reaction were resolved in 8% denaturing PAGE.

Analysis of protein composition of RP_c, RP_o and RP_{el} complexes

RP_c, RP_o and RP_{el} were formed as described above but the volumes of the reactions were increased 10 times. The DNA–protein complexes were loaded on 4 ml Sephacryl S-400 columns (Pharmacia). The column was washed with 1 ml of transcription buffer and then 100 µl fractions containing RP_c, RP_o or RP_{el} complexes were collected. The DNA-bound proteins were eluted from the column in the void volume together with the plasmid DNA and thus separated from DNA-free proteins retained on the column. The purified complexes co-eluted with the DNA peak fractions, were resolved in SDS–PAGE and silver stained.

RESULTS

Initiation complexes are formed *de novo* on the *glnAp2* promoter during every round of transcription: footprinting studies

To analyze the mechanism of enhancer action over a large distance, plasmids having 110 bp enhancer–promoter spacing or entirely lacking the enhancer (Fig. 1) were used in the experiments described below. Enhancer-dependent transcription of the *glnAp2* promoter was initially characterized using a single-round transcription assay (10). As expected, transcription from the *glnAp2* promoter requires the presence of all components described previously (core polymerase, promoter DNA, NtrC, NtrB and σ^{54}) and occurs in an ATP- and enhancer-dependent manner (data not shown).

The goal of the initial experiments was to characterize DNA–protein interactions established during transcription initiation from the *glnAp2* promoter. DNase I and $KMnO_4$ footprinting methods (27) were utilized to analyze the structures of RP_c, RP_o and RP_{el} complexes formed on supercoiled template containing the enhancer positioned 110 bp upstream the *glnAp2* promoter (Fig. 2). $KMnO_4$ preferentially modifies single-stranded DNA regions containing thimidines and is used for detection of melted DNA (27). As has been shown previously, the addition of NtrC, NtrB, σ^{54} and core RNA polymerase to the template in the absence of ATP results in the formation of RP_c (30). In RP_c, core RNA polymerase protects the –35 to +1 promoter DNA region from DNase I (Fig. 2, lanes 2 and 6) but promoter DNA remains double stranded and shows only weak sensitivity to $KMnO_4$ (lane 10) as compared with free DNA (lane 9). Addition of ATP induces the formation of a footprint extending from –35 to +25 characteristic for RP_o (lanes 3 and 7) and the appearance of a region of the promoter (at +1 position) highly reactive to $KMnO_4$ (lane 11) indicating that RP_o contains a melted DNA region at the start site (29).

This template contains a U-less cassette corresponding to the first 18 nt in the transcript. In the presence of ATP, CTP and GTP (–UTP reaction) the polymerase was expected to stall at the position where it needs to incorporate UTP (nucleotide 18). In fact, the majority of the elongation complexes were stalled at the position before the next U in the RNA sequence (position +36; Fig. 2, lane 12 and data not shown), presumably due to read-through of the first U. Under these conditions a strong

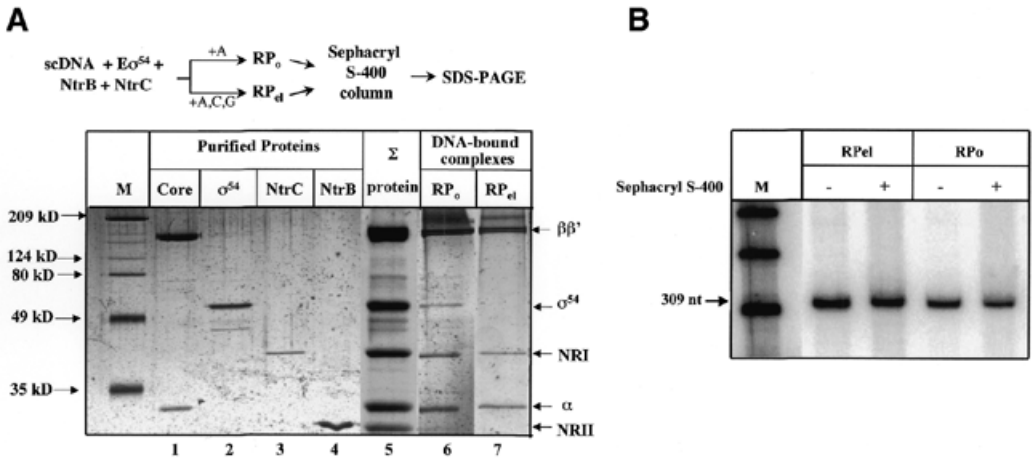

Figure 3. Escape of the RNA polymerase from the promoter is accompanied by dissociation of σ^{54} from DNA. (**A**) RNA polymerase subunit σ^{54} is depleted from early RP_{el}. The experimental strategy for purification of RP_c and RP_o complexes is outlined at the top. The complexes were purified from DNA-free proteins on a Sephacryl S-400 column, separated in an SDS–PAGE and silver stained (lanes 6 and 7). Purified proteins used for preparation of the transcription complexes were of ~95% purity and were loaded as additional markers (lanes 1–4). Total proteins present in the reaction mixture were stained with Coomassie (lane 5). In some experiments NtrC was under-represented in RP_{el} as compared with RP_o suggesting that NtrC was more stably bound to DNA in RP_o. NtrC was not detectable in either of the complexes if column fractionation was conducted in the presence of excess competitor DNA containing strong NtrC binding site (data not shown). M, protein molecular mass markers. (**B**) Functional RP_o and RP_{el} complexes survive Sephacryl S-400 column. RP_o and RP_{el} complexes were analyzed using a single-round transcription assay before (–) or after (+) fractionation on a Sephacryl S-400 column. No DNA-free proteins were added to the reaction after the column.

protection of the downstream region of the top strand (extending from +30 to +60) from DNase I was accompanied by disappearance of the RP_o-specific footprint at the upstream promoter region; a weak RP_c footprint was still detectable at the promoter (Fig. 2, lane 4). This was accompanied by the disappearance of the $KMnO_4$-sensitive RP_o-specific region at the promoter and the appearance of a new sensitive region at position +32 to +36 (lane 12) confirming that RP_{el}s were stalled at position +36. These results indicate that the RNA polymerase quantitatively escapes from the promoter and that the promoter is then occupied by another molecule of RNA polymerase that cannot form RP_o, probably because the downstream promoter DNA is blocked by RP_{el}. Thus, the second molecule of RNA polymerase remains arrested in RP_c. When transcription was conducted in the presence of all NTPs and an excess of competitor DNA, no footprint was detected at the promoter (lane 8). This indicates that another molecule of RNA polymerase cannot bind to the promoter under these conditions, in agreement with previously published data (16).

In summary, the footprinting studies suggest that when RNA polymerase escapes from the *glnAp2* promoter into RP_{el}, both DNase I and $KMnO_4$ footprints disappear from the promoter unless experimental conditions allow binding of another molecule of RNA polymerase to the promoter.

The σ^{54} subunit is displaced into solution during escape of the RNA polymerase from the *glnAp2* promoter

To determine the protein composition of RP_c, RP_o and RP_{el}, the complexes were formed and then purified from DNA-free proteins by gel-filtration on a Sephacryl S-400 (31) allowing purification of functionally active transcription complexes (Fig. 3B). Fractions containing transcriptionally active complexes had the same elution profile as the plasmid DNA template and came out in the void volume of the column. Pooled fractions were analyzed by SDS–PAGE (Fig. 3A). RP_c did not survive the gel-filtration: only NtrC was detected in the gel indicating that the affinity of RP_c to DNA is not very high (data not shown). NtrB protein was quantitatively depleted during the chromatography which provided an internal control for quality of purification from DNA-free proteins. Purified RP_o (Fig. 3A, lane 6) consisted of three protein complexes stably bound to DNA: core RNA polymerase, σ^{54} and NtrC. RP_{el} had the same protein composition but did not contain σ^{54} (lane 7); thus, the σ^{54} subunit is stably associated with DNA only in RP_o.

In summary, both the data on the protein composition of RP_o and RP_{el} (Fig. 3A) and the footprinting data (Fig. 2) are consistent and indicate that in RP_o, $E\sigma^{54}$ and NtrC are stably bound to the promoter and the promoter DNA is partially melted around the +1 region. After RNA polymerase escapes from the promoter and forms RP_{el}, the σ^{54} subunit is displaced from the complex and the promoter DNA is not melted before the next molecule of RNA polymerase binds and RP_o is formed again. Thus, the RP_o complex must form *de novo* during every round of transcription initiation from *glnAp2* promoter. No evidence for the presence of a DNA–protein complex surviving multiple rounds of transcription was obtained by any of the techniques described above.

Lack of functional 'memory' during enhancer action

The structural data described above do not rule out the possibility that there is a functional 'memory' facilitating multiple rounds of transcription. For example, some DNA–protein complexes may remain weakly bound to promoter in RP_{el}, but are not detectable by the methods described in Figures 2 and 3. Or, functional memory could result from alteration of the template DNA during the first round of transcription. If a functional 'memory' exists, the first round of transcription should occur more slowly than the subsequent rounds. This possibility was investigated by comparison of the rates of single- and multiple-round transcription on supercoiled pLR100 template (containing the natural 110 bp enhancer–promoter spacing; Fig. 4). The

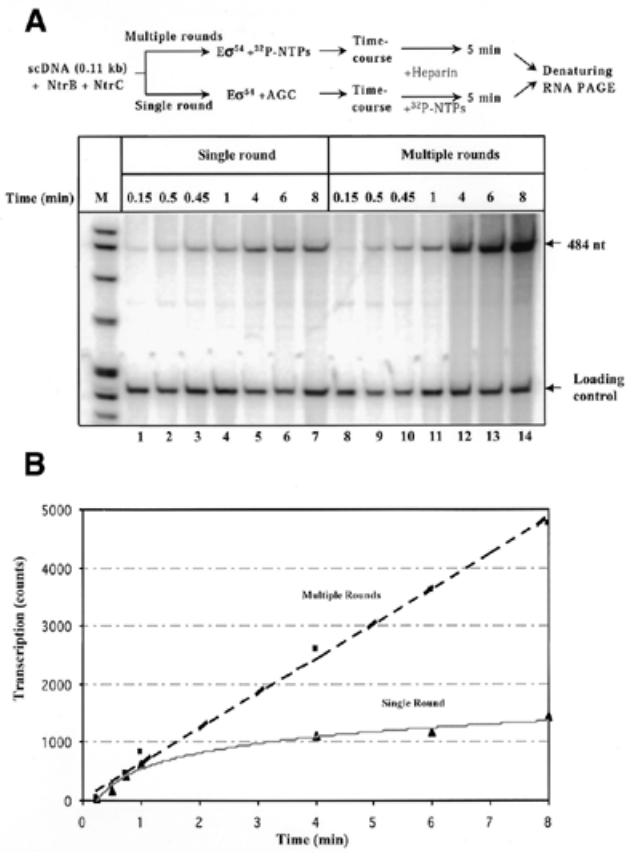

Figure 4. Lack of functional 'memory' during enhancer-dependent activation of the *glnAp2* promoter. (**A**) Time-courses of single-round and multiple-round transcription of supercoiled pLR100 plasmid having 110 bp enhancer–promoter spacing. The experimental strategy for comparison of the rates of single- and multiple-round transcription is outlined at the top. Reaction mixtures were incubated in the presence of all nucleotides (multiple-round) or with ACG mixture only (single-round). Use of an ACG mixture instead of ATP prevents conversion of RP_o back to RP_c and thus allows comparison of single- and multiple-round transcription under similar conditions. The reaction was terminated by adding heparin with subsequent incubation for 5 min. Heparin prevents formation of new initiation complexes but allows completion of already initiated transcripts. Labeled transcripts were analyzed in a denaturing PAGE. The loading control (a 227 bp end-labeled DNA fragment) was added to the reaction mixtures immediately after terminating the reaction. (**B**) Initiation of transcription occurs at similar rates during the first and subsequent rounds of transcription on supercoiled DNA. Quantitative analysis of the data is shown in (A). The intensities of the bands containing 484 nt transcripts were analyzed using a PhosphorImager.

half-time for transcription initiation in the first round was ~1 min and during multiple-round transcription one transcript was synthesized every 2 min (Fig. 4B). Thus, transcription initiation during the first and subsequent rounds occurs with similar rates suggesting that all rounds of transcription are functionally equivalent and that there is no 'memory' facilitating multiple-round transcription.

Previously, we have shown that the efficiencies of transcription of supercoiled templates having an enhancer–promoter spacing of 110 or 2500 bp were not very different (23). In fact, multiple-round transcription of a template with 2.5 kb enhancer–promoter spacing was only ~2-fold less efficient as compared with the template having 0.11 kb spacing (23). Thus, in spite of the fact that under our experimental conditions neither functional nor structural memory were detected at the *glnAp2* promoter during multiple rounds of transcription, the enhancer can work efficiently over a large distance. The data suggest that the 'memory' is not required for efficient enhancer action over a distance.

DISCUSSION

In summary, an experimental system supporting multiple (up to four) rounds of transcription from the *glnAp2* promoter *in vitro* has been established (Fig. 4). Using this system, it has been shown that neither structural nor functional 'memory' is established during the first round of transcription: the first and subsequent rounds are kinetically and structurally identical (Figs 2–4). In our previous studies it has been shown that NtrC-dependent enhancer can efficiently work over a large distance (23). Taken together, the data suggest that the 'memory' is not required for enhancer action over a short or long distance.

It has been shown previously that after RNA polymerase escapes from the *glnAp2* promoter and RP_{el} is formed, a DNase I footprint and DNA melting are still detected at the promoter, suggesting that some protein (presumably the σ^{54} subunit) remains bound there (29). However, analysis of the protein content of the early RP_{el} purified by gel-purification (16) or gel-filtration (31) revealed the absence of the σ^{54} in the complex. In agreement with the latter data, no σ^{54} subunit was detected in the RP_{el} purified by gel-filtration (Fig. 3A). Moreover, DNase I and $KMnO_4$ footprinting studies did not reveal the presence of any footprint at the *glnAp2* promoter after escape of the polymerase from the promoter (Fig. 2). This apparent disagreement of our data and some earlier published results (29) could be explained by the use of different protein preparations. In any case, the lack of the 'memory' does not prevent very efficient enhancer action over different distances and activating of up to four rounds of transcription. Taken together, the data suggest that the structural 'memory' observed in some studies (16,29,31) is not essential for efficient enhancer action. In fact, the protein that remains bound at the promoter was inhibitory for subsequent rounds of transcription (29). In agreement with the structural studies, functional studies revealed no indication of 'memory' facilitating re-initiation after the first round of transcription (Fig. 4). Thus, on supercoiled DNA the first and the subsequent rounds of transcription are structurally and functionally identical (Figs 2–4).

Our previous data suggested that DNA supercoiling greatly facilitates action of the NtrC-dependent enhancer over a large distance probably bringing enhancer and promoter in close proximity (23). In combination with our present results on the lack of 'memory' during enhancer-dependent transcription, the data suggest that DNA supercoiling is a principal factor mediating the action of NtrC-dependent enhancer over a distance, and eliminates the possibility that supercoiling facilitates enhancer action by establishing 'memory' at the promoter (see Introduction).

The above data suggest the following mechanism of action of the NtrC-dependent enhancer (Fig. 5). Before activation, non-phosphorylated NtrC is bound at the enhancer and the holoenzyme forms RP_c at the *glnAp2* promoter both *in vitro* (Fig. 2) (10,16,17) and *in vivo* (15). Transcription activation starts when NtrC is phosphorylated by NtrB protein (32 and

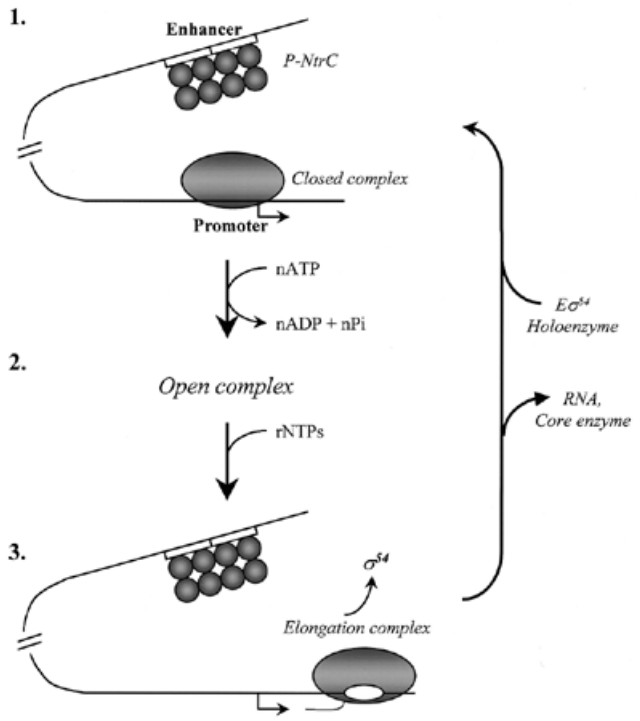

Figure 5. Mechanism of action of NtrC-dependent enhancer during multiple-round transcription of the *glnAp2* promoter. (1) Before transcription of NtrC-dependent genes is induced, the $E\sigma^{54}$ holoenzyme forms a closed complex (RP_c) at the promoter (localized at the –24 to –12 DNA region) but cannot initiate transcription. NtrC is bound to the enhancer (two 17 bp NtrC-binding sites indicated by open boxes) but cannot communicate with the promoter. After induction and phosphorylation by NtrB, NtrC forms homooligomers and interacts with the holoenzyme causing looping out of the intervening DNA (not shown) and ATP-dependent formation of the open complex (RP_o) at the promoter (2). After formation of RP_o, enhancer–promoter interaction is broken and the DNA loop is opened. As the RNA polymerase leaves the promoter (3), the σ^{54} subunit dissociates into solution and the holoenzyme has to re-bind to the promoter and re-establish the interaction with the enhancer for the next round of transcription to occur.

references therein) and NtrC forms higher order homooligomer complexes essential for activation of transcription (intermediate 1; Fig. 5) (11–14). Phosphorylation of the NtrC also activates its ATPase activity required to stimulate conversion from RP_c to RP_o (11–14). Phosphorylated NtrC interacts with the holoenzyme bound at the promoter such that the intervening DNA forms a loop (18,19). This step is greatly facilitated by DNA supercoiling when the enhancer is positioned far from the promoter (23). Once established, enhancer–promoter interaction greatly stimulates the $RP_c \rightarrow RP_o$ transition, the rate-limiting step in the absence of the enhancer (10,16,21,33). When formation of RP_o is completed (intermediate 2), enhancer–promoter interaction is destabilized (19); RP_o is stable and does not require the continued presence of the enhancer for completion of the first round of transcription initiation (16). In the presence of NTPs, the RP_{el} leaves the promoter and the σ^{54} subunit is displaced into solution (intermediate 3), leaving no structural or functional 'memory' at the promoter. Finally, the next molecule of RNA polymerase arrives at the promoter and forms RP_c starting a new transcription cycle (intermediate 1).

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