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# Biochemical Analysis of Enhancer-Promoter Communication in Chromatin

**Yury S. Polikanov**, **Mikhail A. Rubtsov**, and **Vasily M. Studitsky***
*Department of Pharmacology, UMDNJ-Robert Wood Johnson Medical School, 675 Hoes Lane, Piscataway, NJ 08854, USA*

## Abstract

Regulation of many biological processes often occurs by DNA sequences positioned over a large distance from the site of action. Such sequences, capable of activating transcription over a distance, are termed enhancers. Several experimental approaches for analysis of the mechanisms of communication over a distance between DNA regions positioned on the same molecule and, in particular, for analysis of enhancer-promoter communication were developed recently. Most of these methods are technically complicated and not applicable for studies of various important aspects of distant interactions in chromatin. As an alternative, we propose a more efficient and versatile method for the study of enhancer-promoter communication in chromatin using a prokaryotic model enhancer-promoter system that recapitulates most of the key aspects of eukaryotic transcriptional enhancer action (including action over a large distance) both *in vivo* and *in vitro*. Below we describe the application of this highly efficient experimental system to analyze the structural and dynamic properties of chromatin that allow communication between DNA regulatory regions over a distance.

### Keywords

chromatin; nucleosome; enhancer; promoter; transcription

## Introduction

The process of transcription is essential for all living cells. Initiation is the first and in most cases regulated step of transcription. This regulation often occurs by DNA sequences positioned over a large distance from the site of action. These sequences, capable of activating transcription over a distance, are termed enhancers (E).

The unique property of enhancers is their ability to activate target genes over more than 100 kb *in vivo* [1]. Most likely, enhancer action involves direct interaction between proteins bound at the enhancer and target promoter with accompanying formation of a large chromatin loop that includes the intervening DNA [2–4]. Therefore efficient enhancer action over a distance critically depends on structural and dynamic properties of chromatin that are largely unknown. The precise mechanism of enhancer action over a large distance remains unknown, but available data indicates that enhancers adopted a special mechanism for efficient communication with promoters over a large distance [4,5].

* Author to whom the correspondence should be addressed: Tel. (732) 235-5240, Fax (732) 235-4073, e-mail: studitvm@umdnj.edu.

Several experimental approaches for analysis of the mechanisms of communication over a distance between DNA regions positioned on the same molecule and, in particular, for analysis of the mechanism of enhancer-promoter communication were developed recently. First, using the FLP DNA recombination assay [6] as a tool for measuring communication over a distance both *in vivo* and *in vitro* it was shown that chromatin increases the effective DNA flexibility only at short distances. During FLP recombinase-mediated excision, two target sites (FRTs) are bound by FLP, synapsis takes place and recombination results in the excision of the intervening DNA (reviewed in [7]). Using a set of excision substrates with varying distances between their FRT sites ranging from 74 bp to 15 kb, it was shown that FLP-mediated excision allows measuring the efficiency of DNA looping (communication between the FRT sites) on linear DNA both *in vitro* and *in vivo* [6]. However, applicability of this method for studies of communication in chromatin was not investigated. Moreover, communication between the DNA sequences required for recombination and E-P communication may occur by different mechanisms. Thus, recombination can occur between DNA sequences positioned within different, sometimes widely spaced domains of chromatin while E-P communication is largely limited by a single chromatin loop [8,9].

Since the efficiency of intramolecular communication between the ends of linear DNA depends on their local concentration in the vicinity of each other *in cis*, DNA ligation-circularization assay was used as an alternative method for analysis of communication over a distance [10–12]. Although this approach is applicable for studies of communication properties of DNA and was applied to the analysis of communication in chromatin [13], our preliminary data suggest that inter-nucleosomal interactions strongly contribute to the previously observed effects on communication [13] and complicate interpretation of the experiments (Y.S.P., data not shown). Furthermore, the ligation-circularization assay can be applied only to linear DNA molecules and therefore the effects of DNA supercoiling on communication cannot be studied.

To date, there is a very limited number of studies of the mechanism of eukaryotic enhancer action over a distance *in vitro*, primarily because eukaryotic RNA polymerase II-dependent *in vitro* transcription systems are inefficient [14] and rarely recapitulate enhancer action over a distance [15,16]. At the same time, bacterial transcriptional enhancers can work efficiently over a large distance (up to at least 3.5 kb) both *in vivo* and *in vitro* [17–19]. Moreover, pro- and eukaryotic transcriptional enhancers share many key properties [1], and our preliminary data suggest that bacterial enhancers can also work efficiently over a large distance in chromatin environment *in vitro*. The mechanism of action of bacterial transcriptional enhancers has been extensively studied using the *glnAp2* promoter of *E. coli* as a model system [20,21]. This elegant experimental enhancer-dependent system was established and investigated in the laboratories of Drs. Sidney Kustu [22] and Boris Magasanik [23]. Activity of the *glnAp2* promoter is entirely dependent on the NtrC activator-dependent, $\sigma^{54}$-dependent transcriptional enhancer [19,24,25], which participates in regulation of genes involved in nitrogen metabolism [26]. The enhancer is activated by the NtrC protein, which is phosphorylated by the NtrB protein kinase [27,28]. When phosphorylated, enhancer-bound NtrC forms homooligomers, interacts with the $E\sigma^{54}$ holoenzyme and stimulates conversion of the closed ($RP_{closed}$) to the open ($RP_{open}$) initiation complex [19,24,29–32]. During this enhancer-promoter interaction, intervening DNA is transiently looped out [33,34], placing enhancer and promoter in close proximity to each other [35].

Experimental system for studies of the mechanism of enhancer-promoter communication described above is relatively simple, highly efficient, and is very well studied. Transcription is strongly (>100-fold) stimulated by the enhancer and the mechanism of communication can be analyzed both *in vitro* and *in vivo*. Activity of the promoter itself in this system does not depend on the level of negative DNA supercoiling (Y.S.P., unpublished data) that makes possible to analyze communication properties of linear, relaxed or supercoiled DNA, as well

as chromatin templates. The stage of physical interaction between enhancer and promoter is the rate-limiting step for the whole process of enhancer-dependent transcription [18]. Therefore, the overall efficiency of transcript accumulation can serve as a direct measure of E-P communication and allows quantitative analysis of the rate of communication.

Using this experimental technique we have recently shown that action of NtrC-dependent enhancer over a large (2.5 kb) distance is greatly stimulated by negative DNA supercoiling [18]. Moreover, for the first time it was shown that enhancer-blocking insulator-like activity can be recapitulated *in vitro* in a highly purified system using a sequence-specific DNA-binding protein that makes stable DNA loops [17]. This rationally designed insulator recapitulates all key properties of eukaryotic insulators observed *in vivo* [17]. Later, independent studies have suggested that similarly designed regulatory elements have indeed strong insulator activity in mammalian cells *in vivo* [36].

In summary, the NtrC-dependent, enhancer-dependent *glnAp2* promoter is a well-studied, highly efficient experimental system recapitulating many key aspects of eukaryotic transcriptional enhancer action (including action over a large distance) both *in vivo* and *in vitro*. Below we describe application of this highly efficient experimental system to analysis of structural and dynamic properties of chromatin that allow communication between DNA regulatory regions over a distance. Some of the techniques described below are also applicable to analysis of communication between various regulatory regions on histone-free DNA.

## 2. Description of methods

### 2.1 DNA templates for analysis of distant communication between enhancer and promoter

To analyze enhancer action over a large distance in chromatin environment, the 7637 bp pLY10 plasmid was used [18]. It contains the *glnAp2* promoter that is strongly (more than 100-fold) activated by NtrC-dependent enhancer positioned 2.5-kb away from the promoter [18,37]. It was shown previously that DNA supercoiling can greatly (up to 50-fold) facilitate enhancer-promoter (E-P) communication over a large distance on histone-free pLY10, which is the rate-limiting step during distantly activated transcription of the relaxed or linear plasmid [18].

In order to study the distance-dependence of E-P communication on relaxed DNA several additional plasmids having various distances between the enhancer and promoter were constructed. All plasmids were designed to have E-P spacing in the range of 0.5 to 2.5 kb. At E-P distances less than 0.5 kb the effects of local DNA twisting could strongly modify the efficiency of transcription and complicate the interpretation of the experiments.

### 2.2 Preparation of DNA templates having different degrees of negative supercoiling

Samples of pLY10 plasmid supercoiled to different extents were prepared and used as markers in topological analysis (Fig. 1). To obtain samples of the pLY10 plasmid that are negatively supercoiled to various extents, the supercoiled plasmid (10 μg/ml) was incubated in the presence of Chicken Blood Extract (CBE, [38]) possessing strong DNA topoisomerase I activity and different concentrations of chloroquine. Chloroquine is an intercalator that binds to DNA, causes its partial unwinding and introduces positive supercoiling. Therefore DNA molecules relaxed by DNA topoisomerase I in the presence of chloroquine become negatively supercoiled after removal of DNA-bound chloroquine. The extent of negative DNA supercoiling depends on the concentration of chloroquine during relaxation. The CBE:DNA ratio must be determined empirically for each batch of CBE to achieve complete relaxation of the DNA. The present studies were performed with 0.1 μl of the CBE per 1 μg of pLY10 DNA. The reactions were conducted in buffer containing 200 mM NaCl, 20 mM Tris-HCl (pH 8.0), 0.25 mM EDTA and 5% glycerol for 2 hr at 37ºC in the presence of the following concentrations

of chloroquine (μg per ml): 0 (σ = 0), 50 (σ = −0.01), 100 (σ = −0.024), 200 (σ = −0.032), 300 (σ = −0.042), 500 (σ = −0.050), and 700 (σ = −0.055) (Fig. 1). Superhelical density (σ) shows fraction in % of superhelical coils from total number of helical coils in the plasmid or any linear DNA fragment with immobilized ends. Magnesium ions that are present in the transcription buffer (see below) cause some increase in superhelical densities of supercoiled plasmids by −0.01 and under transcription conditions the samples of pLY10 plasmid had the following σ's respectively: −0.010, −0.020, −0.034, −0.042, −0.052, −0.060 and −0.065. For this reason, to obtain completely relaxed form of the plasmid with σ = 0 under transcription conditions, pLY10 was linearized and then re-ligated in the transcription buffer. The relaxed and supercoiled plasmids were then purified by extraction with phenol/chloroform followed by ethanol precipitation.

This method can be used for small-scale as well as large-scale preparations of plasmids with different levels of supercoiling, but it requires some preliminary work, such as isolation and purification of CBE, its titration and optimization of reaction conditions. Purification of plasmid DNA from *E. coli* at different stages of cell growth could be used as an alternative approach for large scale preparation of plasmids with different levels of supercoiling. However, this method only allows purification of DNA with superhelical densities in the range −0.03 to −0.09, depending on the stage of cell growth and growth conditions, such as ATP/ADP ratio, temperature and/or osmotic status [39].

### 2.3 Topological analysis of supercoiled DNA

Topological analysis of all forms of pLY10 plasmid having different degrees of supercoiling was performed in order to precisely determine their superhelical densities (σ). This method allows identification of different topoisomers of one plasmid by differences in their electrophoretic mobilities. Although topoisomers of the moderately supercoiled plasmid can be resolved by ordinary agarose gel electrophoresis, various strongly supercoiled topoisomers have the same electrophoretic mobility that does not allow separation of different topoisomers in the gel. For negatively supercoiled DNA, this limitation can be overcome by including an intercalating agents, such as chloroquine or ethidium bromide, in the electrophoresis gel [40, 41]. As mentioned above, these agents cause partial relaxation of negative or introduces weak positive supercoiling of the topoisomers that results in shifting of the distribution centers of topoisomers towards less negatively supercoiled and/or weakly positively supercoiled forms.

To resolve the pLY10 topoisomers, all obtained samples were loaded on a series of four 0.6% agarose gels in 1x TAE buffer containing 0.9, 1.5, 5 or 10 μg per ml of chloroquine, respectively (Fig. 1). Electrophoresis was carried out at 4ºC in cold room for 15 hours at 1.5 volts/cm in 20x20x0.5 cm agarose gels to achieve resolution of all topoisomers on all four gels. Next, the gels were stained with ethidium bromide at 0.5 μg per ml for 30 minutes to visualize the bands and washed three times with distilled water for 1 hour each time to remove traces of unbound ethidium bromide and reduce the background. The value of σ for each obtained sample was determined by direct counting of the bands on the gel using reference points. Relaxed pLY10 sample (σ = 0) was used as a reference point for the first gel (0.9 μg per ml of chloroquine). pLY10 samples with σ = −0.01, −0.032 and −0.0042 were used as reference points in the case of other agarose gels containing 1.5, 5 and 10 μg per ml of chrloroquine, respectively (Fig. 1). Since CBE (topoisomerase I) changes the number of supercoils for any circular topoisomer one-by-one, it is possible to determine the absolute number of supercoils present in a given molecule. Each sample was represented by a group of topoisomers having normal distribution. For each sample, the center of distribution of topoisomers was identified and scored as number of supercoils. The superhelical density (σ) was calculated using the following formula:

$$\sigma = Y / X$$

where Y is number of supercoils and X is the number of DNA helical turns (X = plasmid length in bp/10.5 bp per turn).

### 2.4 Assembly of chromatin on a supercoiled pLY10 template

*In vitro* reconstitution of chromatin on supercoiled pLY10 template was conducted at various molar ratios of purified chicken erythrocyte core histones [42] to DNA by the step dialysis method [43,44]. Reconstitution of chromatin was conducted in 120 μl aliquots at 10 nM (50 μg/ml) DNA concentration and 0 nM, 125 nM, 200 nM, 375 nM and 475 nM of each of the four core histones to achieve 0%, 25%, 40%, 75% or 95% efficiency of chromatin assembly, respectively. The percent of histone loading in each case was measured (see below) and then calculated assuming that one single nucleosome occupies 150 bp and that the maximal possible number of nucleosomes which pLY10 plasmid can accommodate is ~50 (7637 bp/150 bp per nucleosome).

DNA and core histones were mixed in buffer containing 2 M NaCl, 10 mM Tris HCl (pH 7.5), 0.2 mM EDTA, 0.1% NP40 and 5 mM β-mercaptoethanol, transferred into small dialysis bags and placed in the first dialysis buffer, which is equal to the buffer in the reconstitution mixture. Each step of dialysis was performed at 4°C in cold room in glass beakers and on magnetic stirrer for 2 hours in at least 1000-fold excess volume of buffers, containing 10 mM Tris HCl (pH 7.5), 0.2 mM EDTA, 0.1% NP40 and 5 mM β-mercaptoethanol and decreasing concentrations of salt (2.0 M, 1.5 M, 1.0 M, 0.75 M, 0.5 M, 0 M NaCl, respectively). The last step of dialysis (0 M NaCl) was continued overnight.

### 2.5 Characterization of assembled chromatin

The efficiency of chromatin assembly was monitored using four assays: analysis of the level of constrained DNA supercoiling in chromatin [41], quantitative restriction enzyme sensitivity assay [45], digestion with micrococcal nuclease and transmission electron microscopy [46].

**2.5.1 Analysis of the level of constrained DNA supercoiling on chromatin templates—**In order to accurately count the number of nucleosomes on each chromatin template the level of constrained DNA supercoiling was determined. This method is based on the observation that each nucleosome constrains one superhelical coil of DNA [41] and therefore the average number of assembled nucleosomes can be calculated if the level of constrained DNA supercoiling (σ or the linking number difference) is known. After chromatin reconstitution the templates can have some residual unconstrained DNA supercoiling in the inter-nucleosomal spacer DNA, that unlike constrained DNA supercoiling has not been immobilized on the surface of the histone octamer during assembly and which depends on the initial degree of supercoiling of the plasmid DNA and on the efficiency of assembly (Fig. 2). This unconstrained DNA supercoiling has to be removed before analysis of constrained DNA supercoiling.

To determine the sign and the level of constrained DNA supercoiling, chromatin preparations were relaxed to completion in the presence of CBE (topoisomerase I) to relieve any unconstrained DNA supercoiling. Relaxation of chromatin was performed by incubation at 50 μg/ml with CBE (0.1 μl of the CBE per 1 μg of DNA) according to the protocol described in the section 2.2 (Fig. 3). Next, DNA was isolated and its topological analysis (section 2.3) was performed to determine the number of supercoils for each chromatin template that is equal to the number of nucleosomes that were initially bound to the same DNA (Fig. 4).

**2.5.2 Calculations of unconstrained DNA supercoiling present in chromatin templates—**The density of the unconstrained DNA supercoiling ($\sigma_{unconstr}$) on linker DNA that remains histone-free after chromatin assembly was calculated as described [47]. The total

number of histone-free DNA helical turns (H) and the total number of unconstrained DNA supercoils (S) were calculated using the values of σ determined for the original DNA preparations and for the relaxed chromatin samples, and assuming that each nucleosome constrains one negative superhelical coil and occupies 150 bp of DNA [48]. Therefore the level of unconstrained DNA supercoiling (σ) remaining after chromatin assembly on the histone-free part of the DNA depends on the initial level of DNA supercoiling and on the efficiency of the assembly, has corresponding (negative) sign and can be easily calculated using the following formula:

$$\sigma_{\text{unconstr}} = S / H = \frac{Z + X \times Y / 150}{(X - X \times Y) / 10.5}$$

X – length of the plasmid, bp

Y – fraction of the plasmid assembled into nucleosomes (% of assembly/100)

Z = σ x X/10.5 (negative if σ is negative) – number of unconstrained supercoils present on *histone-free* plasmid

S = Z + X x Y/150 – total number of *unconstrained* supercoils (negative if σ is negative)

H = (X – X x Y)/10.5 – total number of DNA helical turns present on histone-free part of the plasmid

**2.5.3 Restriction enzyme sensitivity assay—**The restriction enzyme sensitivity assay is based on the observation that chromatin assembly results in strong protection of nucleosome-covered DNA from digestion with restriction enzymes [45,49]. Cutting sites of the restriction enzymes should be chosen in such a way that products of complete digestion of the plasmid would have different lengths in order to make possible their separation on the agarose gel and further analysis. Restriction enzyme digestion was performed on 750 ng of DNA or chromatin templates (20 μg/ml), which were incubated in the presence of an excess of *Dra*I (4 sites) and *Bgl*II (1 site) restriction endonucleases (10 units of each) in NEBuffer 2 (New England Biolabs) at 37ºC for 2 hours. After incubation, the DNA was purified by phenol/chloroform extraction followed by ethanol precipitation and analyzed by electrophoresis in 1% agarose gel in 1x TAE buffer. The intensities of the bands in the gel were quantified using the OptiQuant software (Perkin Elmer). The loading was adjusted to guarantee that intensities of the bands were in linear range of the measuring device.

The intensities of the bands corresponding to the final products of digestion are decreased as the efficiency of chromatin assembly is increased (Fig. 5B). The extent of chromatin assembly is directly proportional to the decrease in the intensity of the bands. At least in the case of pLY10 the partial digestion patterns of histone-free DNA (Fig. 5C) are very similar to the patterns obtained after extensive chromatin digestion (Fig. 5B) indicating that all analyzed sites were equally sensitive to the restriction enzymes in chromatin and DNA, suggesting that nucleosomes are randomly positioned on plasmid DNA. As expected, at 95% chromatin assembly DNA becomes almost completely resistant to digestion with the restriction enzymes, suggesting almost full occupancy of DNA by nucleosomes (Fig. 5B).

**2.5.4 Micrococcal nuclease sensitivity assay—**The assay for monitoring nucleosome assembly described below is based on the fact that nucleosome-covered DNA is strongly resistant to digestion with micrococcal nuclease. As a result of such digestion ~150-bp DNA fragments are produced. Micrococcal nuclease digestion was conducted on 250 ng of DNA or chromatin templates (10 μg/ml) which were incubated in the presence of increasing concentrations of micrococcal nuclease (4, 8, 16, 32 and 64 units per ml) in the buffer containing

20 mM Tris-HCl (pH 8.0), 40 mM KCl, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 1 mM β-mercaptoethanol and 0.1 mg/ml BSA. Reactions were terminated by addition of EDTA to 20 mM final concentration, followed by phenol/chloroform extraction and ethanol precipitation. Purified DNA fragments were end-labeled using T4 polynucleotide kinase and [$\gamma$-$^{32}$P]-ATP, purified by phenol chloroform extraction followed by ethanol precipitation and analyzed by non-denaturing PAGE. The non-denaturing 4.5% acrylamide gel (39:1) was prepared with buffer containing 20 mM HEPES-Na (pH 8.0), 0.2 mM EDTA and 5% glycerol. Electrophoresis was carried out in 20 mM HEPES-Na (pH 8.0)/0.2 mM EDTA buffer at 3 volts/cm for 2 hours. Next, the gel was transferred to 3MM Whatman paper, covered with polyethylene wrap and dried for 30 minutes at 80°C. PhosphorScreen (Perkin Elmer) was placed above the dried gel, exposed overnight and scanned on Cyclone (Perkin Elmer). A representative example is shown in Fig. 6.

The results obtained by different assays are consistent and suggest that the majority of core histones were properly assembled into chromatin and the expected numbers of randomly positioned nucleosomes were formed on the pLY10 DNA.

## 2.6 In vitro transcriptional assays

**2.6.1 Single-round assay for enhancer-dependent in vitro transcription—**Single- and multiple round transcription assays are useful for analysis of different aspects of enhancer action over a distance [18,19,25,37]. The single-round transcription assay is particularly useful for the analysis of kinetic aspects of enhancer action. It is based on the observation that pre-formed open initiation complex formed at the promoter ($RP_{open}$) is stable in the presence of heparin, however, its formation *de novo* is strongly inhibited [50]. Moreover, heparin disrupts nucleosomes, so that they are present only during initiation step, but not during elongation.

The templates are pre-incubated with all proteins (holoenzyme, NtrB & NtrC) without ATP to allow binding of the polymerase and the NtrC activator to DNA. The holoenzyme forms a closed complex at the promoter but cannot initiate transcription and cannot interact with DNA-bound NtrC activator. Since both RNA polymerase and the enhancer-binding protein (NtrC) are pre-bound to DNA, measurements of the rate of enhancer-promoter communication are not complicated by the processes of establishing DNA-protein interactions [18]. Then ATP is added to the reaction for various time intervals to allow E-P communication (this is usually the rate-limiting step in the reaction when the E-P distance is large [18]). This results in phosphorylation of NtrC activator by NtrB protein kinase and P-NtrC interaction with the holoenzyme causing looping of the intervening DNA and ATP hydrolysis-dependent formation of the open complex at the promoter. After formation of the open complex the E-P interaction does not persist. Then the reaction is chased in the presence of heparin and labeled NTPs. As the RNAP leaves the promoter, the $\sigma^{54}$ subunit dissociates into solution.

Samples of chromatin and histone-free DNA can be relaxed with CBE before transcription according to the protocol described in section 2.5.1, so that transcriptional analysis can be performed either on supercoiled or on relaxed chromatin templates (Fig. 3). Conditions for *in vitro* transcription were optimized for maximal utilization of the chromatin templates as well as histone-free pLY10. Transcription was conducted in 50-μl aliquots in the transcription buffer (TB) containing 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 at 1 nM DNA or chromatin concentrations and 10 nM core RNA polymerase, 300 nM $\sigma^{54}$, 120 nM NtrC, and 400 nM NtrB (Fig. 7). First, all components were mixed together in 1x TB and total volume of 40-μl, then the reaction mixture was incubated for 15 min at 37°C to form the closed initiation complex ($RP_{closed}$). Next, 5 μl of 40 mM ATP in 1x TB were added to the reaction to 4 mM final concentration, and the reaction was incubated at 37°C for 1 more minute (or for different time intervals, see below) to form the open initiation complex ($RP_{open}$) which was ready to begin elongation. Then a

mixture of all four ribonucleotide-triphospates (4 mM each) in 1x TB with 2.5 μCi of [α-$^{32}$P]-GTP (3000 Ci/mmol) and 2 mg/ml heparin was added to the reaction to start transcript elongation. The reaction was continued at 37°C for 15 minutes and terminated with an equal volume of phenol/chloroform (1:1). A 254-bp end-labeled DNA fragment was added to the mixture as a loading control. Finally, samples were precipitated with ethanol, dissolved in formamide loading solution, denatured at 95°C for 5 minutes, cooled on ice and separated on 8% denaturing polyacrylamide gel (19:1) containing 0.5x TBE buffer and 8 M urea. Electrophoresis was carried out at 2000 volts and no more than 50 watts for approximately 1 hour until the bromphenol blue front reached the bottom of the gel. Next, the gel was transferred to a filter paper, covered with polyethylene wrap and dried for 30 minutes at 80°C. PhosphorScreen (Perkin Elmer) was placed above the dried gel, usually exposed overnight and scanned on Cyclone (Perkin Elmer). The data was analyzed using the OptiQuant software (Fig. 8).

**2.6.2 Analysis of the rate of enhancer-promoter communication—**The rates of enhancer-promoter communication on supercoiled or relaxed chromatin templates can be compared quantitatively using the single-round transcription assay. In this case, communication is also initiated by adding ATP after pre-formation of $RP_{closed}$ and NtrC-DNA complexes. This approach is similar to the one described in the previous section, but ATP should be added for various time intervals (0, 1, 2, 4, 8, 16, 32 minutes) to allow E-P communication for different times.

## 3. Concluding Remarks

To our knowledge, this is a first set of techniques allowing systematic analysis of the mechanism of communication over a large distance in chromatin *in vitro*. The combination of experimental approaches described above allows a detailed, quantitative and comprehensive molecular description of the mechanism of communication between distantly positioned DNA regions in chromatin and analysis of chromatin properties that are relevant for this process. Some of the techniques described here could be applied to the analysis of the mechanism of regulation of transcription and related processes (such as DNA recombination, repair and replication) involving enhancer action and communication over a distance.

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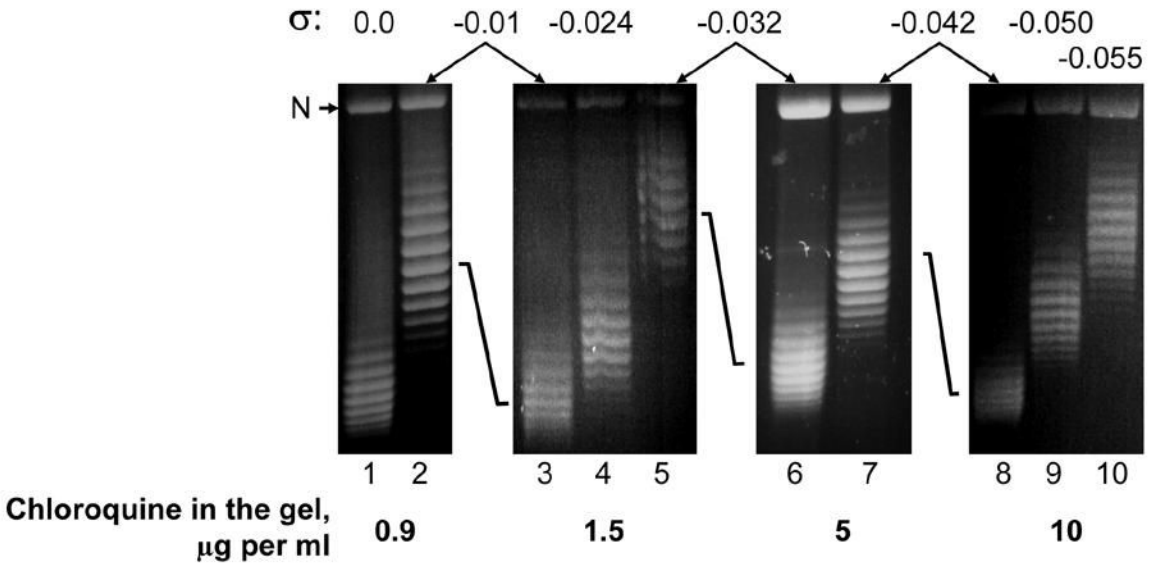


**Figure 1.**

Preparations of the pLY10 plasmid having various values of superhelical densities (σ). Analysis by agarose gel electrophoresis in the presence of various concentrations of chloroquine (0.9, 1.5, 5 and 10 μg per ml, panels from left to right, respectively). Topoisomers having identical superhelical densities on different gels are connected by Z-shaped lines. Average DNA supercoiling densities (σ) are indicated above each lane. N – nicked DNA.

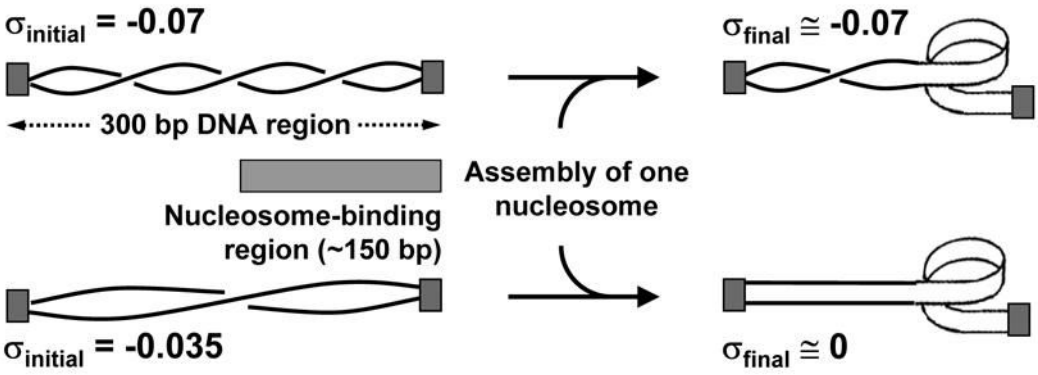


**Figure 2.**

The level of unconstrained DNA supercoiling present after chromatin assembly depends on the initial level of DNA supercoiling and on the number of assembled nucleosome cores. Each nucleosome core constrains one negative DNA superhelical coil and occupies a discrete DNA region (~150 bp). Therefore depending on the initial superhelical density of DNA (σ), unconstrained negative DNA supercoiling can be preserved (top, initial σ = −0.070) or relieved (bottom, initial σ = −0.035) after nucleosome assembly. The drawing corresponds to 50% nucleosome assembly level.

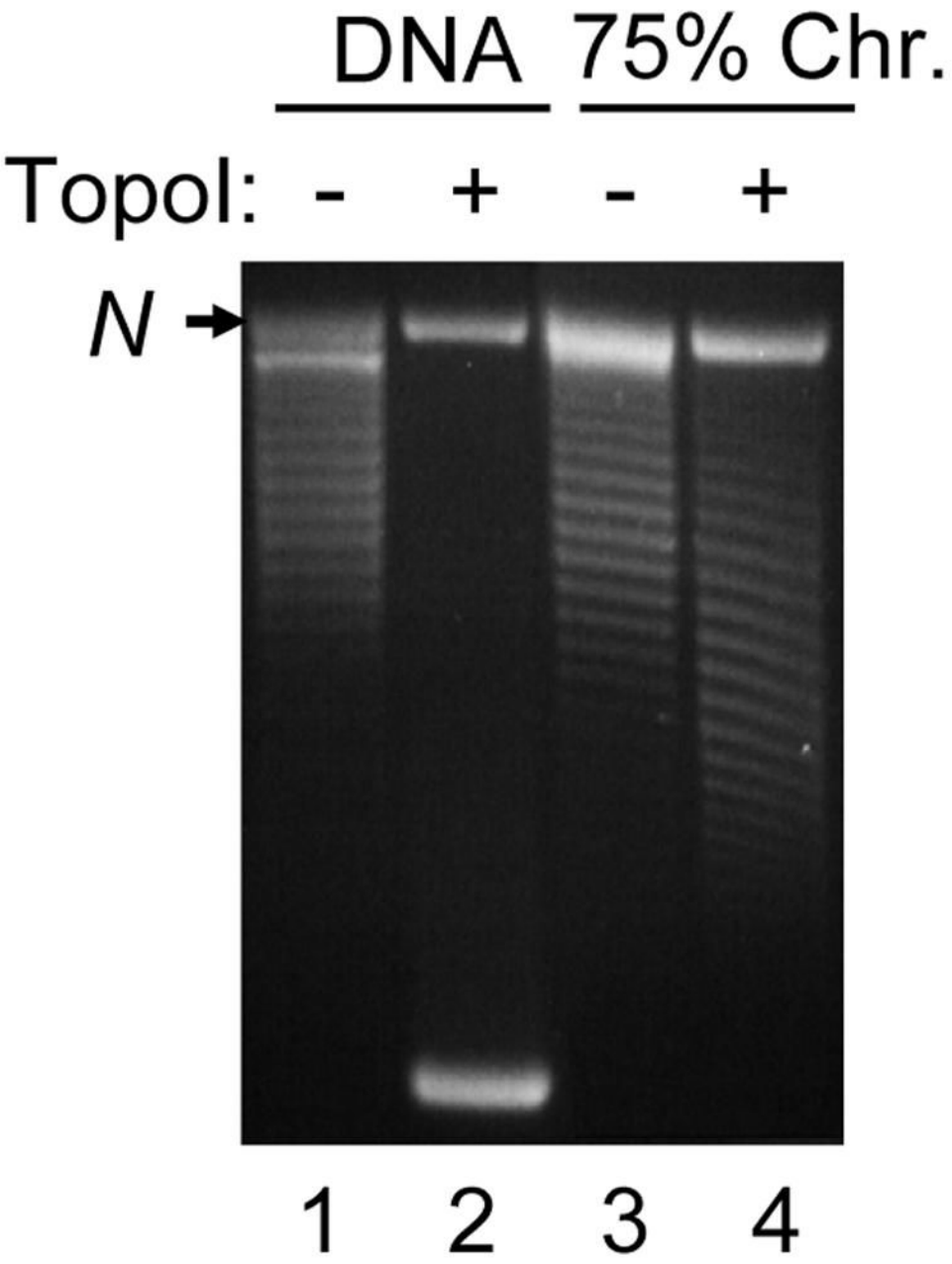


**Figure 3.**
An example of DNA (lanes 1 and 2) or 75% chromatin (lanes 3 and 4) relaxation using topoisomerase I. After relaxation the DNA was isolated and analyzed by agarose gel electrophoresis in the presence of 10 μg per ml of chloroquine. The efficiency of relaxation of the unconstrained DNA supercoiling by treatment with topoisomerase I was monitored in each experiment and in all cases was complete: incubation with much higher concentrations of topoisomerase I and for longer time did not change the final levels of DNA supercoiling or transcriptional activities of the templates.

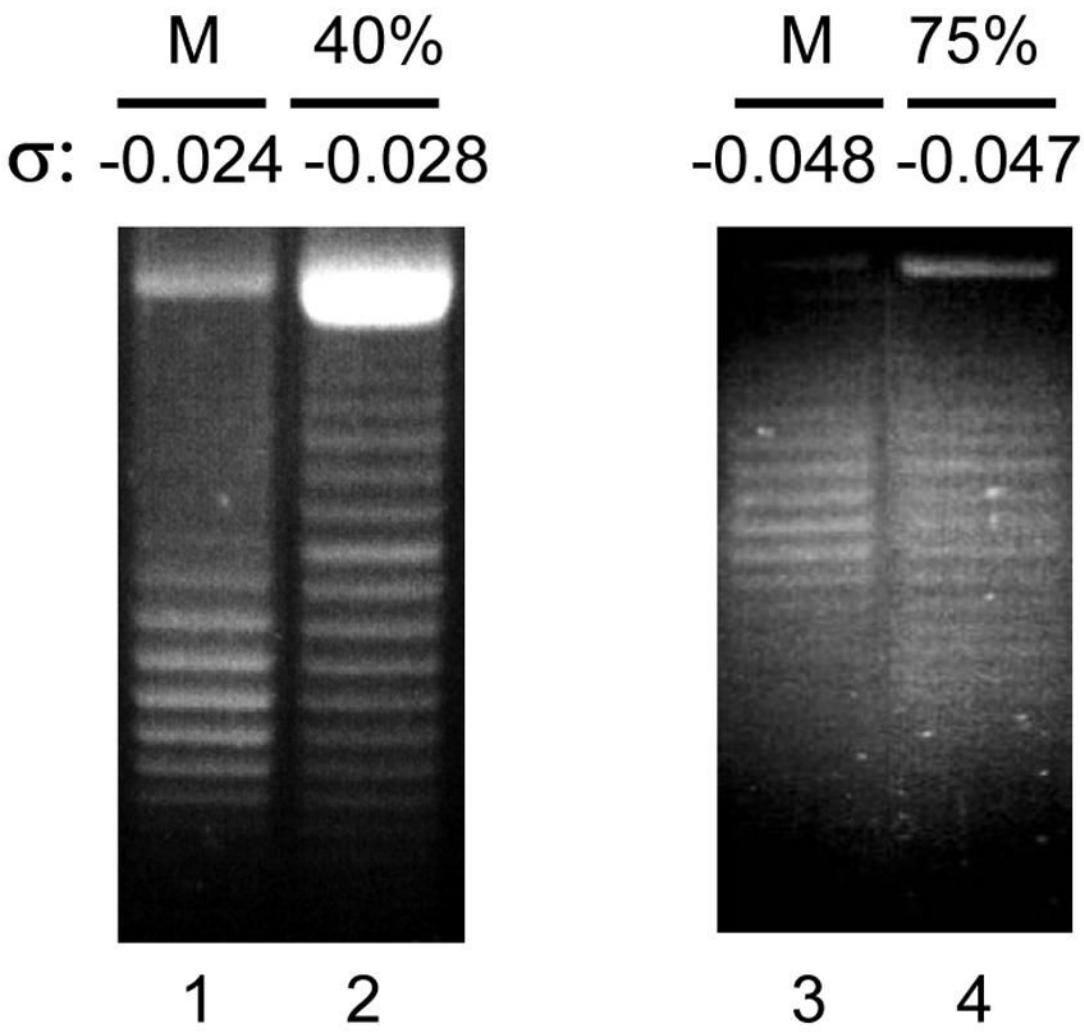


**Figure 4.**
Analysis of the level of constrained DNA supercoiling in chromatin reconstituted on pLY10 plasmid having initial σ = −0.055. Chromatin samples (histone loading 40 or 75%, as indicated) were incubated in the presence of topoisomerase I, DNA was purified and analyzed in a 0.6% agarose gel containing 1.5 (lanes 1 and 2) or 8 (lanes 3 and 4) μg per ml chloroquine. M – preparations of pLY10 plasmid with indicated linking number differences.

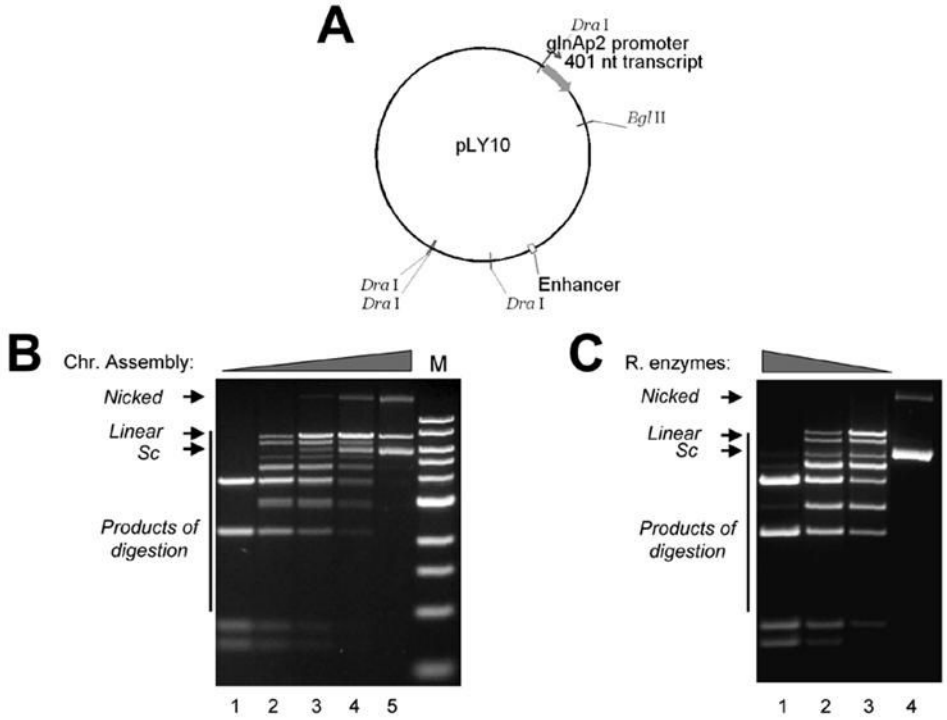


**Figure 5.**

Characterization of chromatin templates using the restriction enzyme sensitivity assay. A. Restriction map of the pLY10 template. The *glnAp2* promoter and NtrC-dependent enhancer are positioned 2.5 kb from each other. Positions of sites for restriction enzymes are indicated. B. Negatively supercoiled pLY10 plasmid ($\sigma = -0.055$) was assembled into chromatin at 0, 25, 40, 75 and 95% histone loading (lanes 1 to 5, respectively) and digested with an excess of restriction enzymes *Dra*I and *Bgl*II. Purified DNA was analyzed by 1% agarose gel electrophoresis. Analysis of purified DNA in 1% agarose gel. Note that the increase of histone:DNA ratio results in progressively better protection of the template DNA from the enzymes and that at 95% chromatin assembly DNA becomes almost completely resistant to digestion with the restriction enzymes, suggesting almost full occupancy of DNA by nucleosomes. M – 1-kb DNA ladder (NEB). C. Partial digestion of histone-free pLY10 DNA with *Dra*I and *Bgl*II restriction enzymes. The pLY10 plasmid was digested in the presence of limiting amounts of restriction enzymes. The amounts of the enzymes were selected to digest DNA to the extents approximately corresponding to the extents of digestion of chromatin preparations shown in Fig. 5B. Note that partial digestion patterns of histone-free DNA (lanes 2 and 3) are very similar to the patterns obtained after extensive chromatin digestion (Fig. 5B, lanes 2, 3 and 4) indicating that all analyzed sites were similarly sensitive to the restriction enzymes in chromatin and DNA, suggesting that nucleosomes are randomly positioned on plasmid DNA.

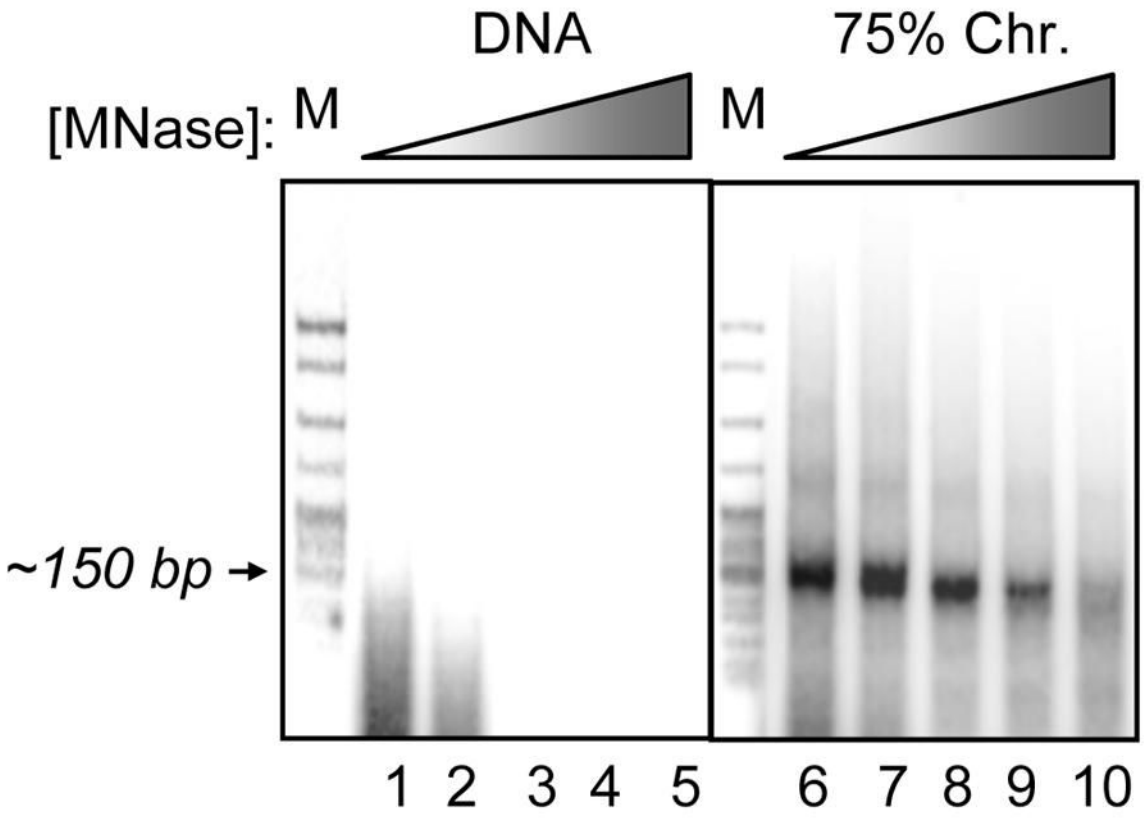


**Figure 6.**

Analysis of 75% chromatin using micrococcal nuclease (MNase). Supercoiled pLY10 plasmid DNA (lanes 1 to 5) or 75% chromatin (lanes 6 to 10) were incubated in the presence of increasing concentrations of micrococcal nuclease (4, 8, 16, 32 or 64 U per ml). After digestion DNA was purified, end-labeled with T4 polynucleotide kinase using [$\gamma$-$^{32}$P]-ATP and analyzed by non-denaturing PAGE. Note appearance of the indicative ~150-bp DNA fragment partially protected from MNase during digestion of chromatin, but not DNA. M – end-labeled pBR322-*Msp*I digest.

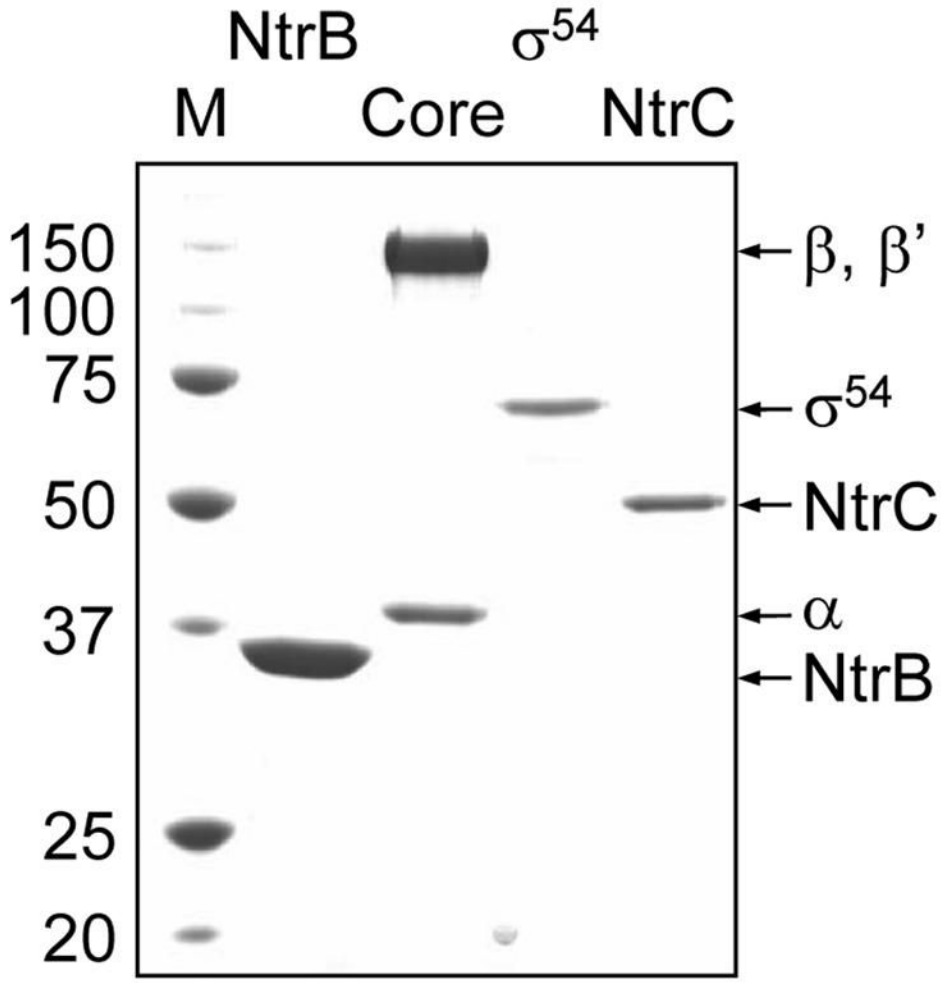


**Figure 7.**
Analysis of purified proteins used for reconstitution of enhancer-dependent transcription *in vitro* system. Proteins were analyzed by 10% PAGE and stained with Coomassie blue. Mobilities of proteins are indicated on the right. M – protein molecular mass marker (Bio-Rad).

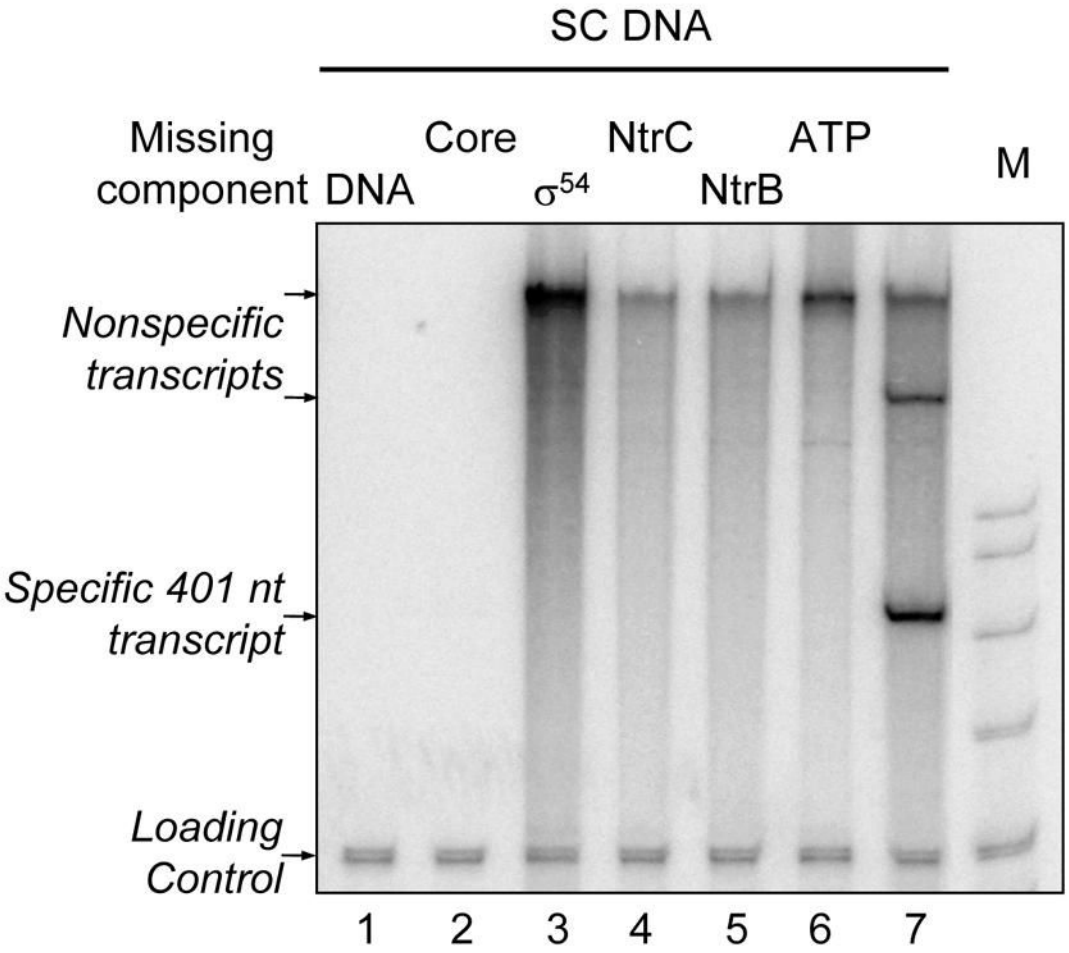


**Figure 8.**

Analysis of *glnAp2* promoter activation by the NtrC-dependent enhancer using the single-round transcription assay. Negatively supercoiled pLY10 plasmid having 2.5-kb enhancer-promoter spacing was transcribed as specified in the text. The reactions contained all components required for enhancer-dependent transcription (lane 7), or one of the components was missing as indicated above each lane (lanes 1 to 6). Note that unlike non-specific transcripts, which appear when core RNA polymerase and a plasmid template are added to the reaction, specific transcript was generated only in the presence of the full set of proteins and ATP. M – end-labeled pBR322-*Msp*I digest.