Function of a Bacterial Activator Protein That Binds to Transcriptional Enhancers

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The nitrogen regulatory (NtrC) protein of enteric bacteria, which binds to sites that have the properties of transcriptional enhancers, is known to activate transcription by a form of RNA polymerase that contains the NtrA protein (σ^{54}) as sigma factor (referred to as σ^{54}-holoenzyme). In the presence of adenosine triphosphate, the NtrC protein catalyzes isomerization of closed recognition complexes between σ^{54}-holoenzyme and the *glnA* promoter to open complexes in which DNA in the region of the transcription start site is locally denatured. NtrC is not required subsequently for maintenance of open complexes or initiation of transcription.

TRANSCRIPTIONAL ENHANCER SEQUENCES FOR EUKARYOTIC genes are binding sites for regulatory proteins. Enhancers lie at a distance from transcriptional start sites—either upstream or downstream—and the regulatory proteins that bind to them activate (or inhibit) transcription (*1*). The precise functions of activator proteins that bind to eukaryotic transcriptional enhancers have not yet been determined, largely because the targets for these activator proteins are not known.

During the past several years sequences analogous to transcriptional enhancers have been identified in prokaryotes (*2–7*). Those that are best studied serve as binding sites for the nitrogen regulatory protein NtrC (*8*) (also called GlnG and NRI) and the nitrogen fixation regulatory protein NifA, which activate transcription by a holoenzyme form of RNA polymerase that contains the *ntrA* product (σ^{54}) as sigma factor (*9–11*). Bacterial sigma factors confer on core RNA polymerase the ability to recognize promoters—sequences that lie just upstream of transcriptional start sites; each holoenzyme (particular sigma factor + core) recognizes a different class of promoter (*12, 13*). NtrC is known to be required prior to completion of a 32-nucleotide (nt) transcript from the promoter for glnA (*9*), the structural gene encoding glutamine synthetase. We now demonstrate that the NtrC protein from *Salmonella typhimurium* is required for isomerization of the initial recognition complex between σ^{54}-holoenzyme and the glnA promoter (closed complex) to a second pre-initiation complex in which the DNA strands are denatured in the region of the transcription start site (open complex); this isomerization requires adenosine triphosphate (ATP).

Stable complexes between σ^{54}-holoenzyme and the glnA promoter. We have used a purified in vitro transcription system to define the role of NtrC in formation of stable complexes between σ^{54}-holoenzyme and the glnA promoter. Various combinations of purified components—core RNA polymerase, σ^{54}, and NtrC—were added to a plasmid template carrying the glnA promoter–regulatory region of *S. typhimurium* upstream of a strong transcriptional terminator. To simplify studies of transcription initiation we used mutant forms of this protein, $NtrC^{con}$ [NtrC (constitutive)], that activate transcription in the absence of the nitrogen regulatory protein NtrB (*9, 14, 15*), a protein kinase that is required to phosphorylate wild-type NtrC (*16, 17*). Although wild-type NtrC can bind to its sites in the glnA promoter region in the absence of NtrB, it cannot activate transcription (*14*).

As demonstrated in protection assays (*14, 15*), σ^{54}-holoenzyme binds to the glnA promoter in the absence of other factors. However, this interaction is disrupted by treatment with the polyanion heparin (*18*), which competes with the template for polymerase binding (*19, 20*). Formation of heparin-resistant complexes is dependent upon $NtrC^{con}$ and ATP in addition to σ^{54}-holoenzyme. When all of these components were incubated with the template, the stable heparin-resistant complexes that formed could be detected by their ability to give rise to transcripts when heparin

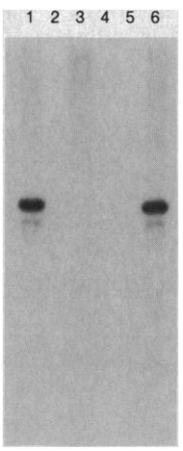

Fig. 1. Requirements for the formation of heparin-resistant complexes between σ^{54}-holoenzyme and the *glnA* promoter. In the complete reactions (lanes 1 and 6), 6 n*M* plasmid template (pJES109, which carries the entire promoter-regulatory region for *glnA* including binding sites for NtrC) (*14, 15*), 40 n*M* core RNA polymerase from *E. coli*, 48 n*M* σ^{54} from *S. typhimurium*, 100 n*M* $NtrC^{con}$ (*ntrC610*) (*14*), and 4 m*M* ATP were present in transcription buffer (see below) before heparin treatment (total volume 25 μl). Components were added to transcription buffer in the order indicated, and reactions were initiated with ATP. In reactions 2 to 5 individual components were omitted before heparin treatment and added immediately afterward; components withheld were core RNA polymerase (lane 2), σ^{54} (lane 3), $NtrC^{con}$ (lane 4), or ATP (lane 5). The reaction mixtures were incubated at 37°C for 8 minutes before heparin (to 50 μg/ml final concentration) and the remaining three nucleotides [GTP and UTP to 0.4 m*M* final concentration, CTP to 0.1 m*M*, and 5 μCi of α-^{32}P–labeled CTP (Amersham, 10 mCi/mmole)] were added simultaneously (5 μl). Transcription was allowed to proceed for 10 minutes at 37°C, and then 20 μg of carrier transfer RNA was added and transcripts were precipitated with ethanol; transcripts were analyzed by electrophoresis in 8 percent sequencing gels (*60*) and visualized by autoradiography at −70°C [Kodak XAR5 or Dupont Cronex film with intensifying screens (Dupont)]. The expected 516-nt transcript (*14*) originating at the *glnA* promoter and ending at a strong T7 terminator (*61*) is seen in lanes 1 and 6. Transcription buffer (*p*H 8.0) contained 50 m*M* tris acetate (*p*H 8.0), 100 m*M* potassium acetate, 8 m*M* magnesium acetate, 27 m*M* ammonium acetate, 1 m*M* dithiothreitol, and 3.5 percent (w/v) polyethylene glycol (6000 to 8000; Sigma). Core RNA polymerase was purified by ammonium sulfate precipitation (55 percent saturation), ion-exchange and affinity (heparin) chromatography, and molecular sizing and σ^{54} was purified by similar steps from an overproducing strain. Both preparations were more than 95 percent pure (*21*).

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and the remaining three nucleotides were added (Fig. 1, lanes 1 and 6). If any of the required components was omitted before the heparin treatment and was added just afterward, essentially no stable complexes were formed (Fig. 1, lanes 2 to 5). The formation of heparin-resistant complexes showed a sigmoidal dependence on ATP concentration with half-maximal response at approximately 0.8 m*M* (*21*).

The ATP requirement for stable complex formation. We eliminated two possible roles for ATP in the formation of heparin-resistant complexes between σ^{54}-holoenzyme and the glnA promoter: ATP does not confer heparin-resistance either by allowing phosphorylation of $NtrC^{con}$ or by allowing transcription to initiate. We tested the first possibility because $NtrC^{con}$ proteins are considerably more active if they are phosphorylated, even though they do not require phosphorylation by NtrB to activate transcription (*6, 15*). We used a direct assay (*16, 17*) to show that there was no detectable kinase activity in the $NtrC^{con}$ preparations (*21*). We then showed that a contaminating kinase could not have been responsible for $NtrC^{con}$ activity by studying the dependence of formation of heparin-resistant complexes on the concentration of an $NtrC^{con}$ protein (*ntrC610*). When this protein was diluted to concentrations between 12 and 0.8 n*M*, the formation of heparin-resistant complexes decreased in proportion to its concentration (Fig. 2), indicating that only the $NtrC^{con}$ protein was limiting in these reactions. In control reconstitution experiments sufficient NtrB kinase was added to wild-type NtrC, which was otherwise completely inactive (*22*), to allow formation of at least as many heparin-resistant complexes as were formed at high concentrations of $NtrC^{con}$ protein (Fig. 2), and this mixture was then diluted. When the concentration of wild-type NtrC was less than 20 n*M*, the formation of heparin-resistant complexes decreased more than could be accounted for by the dilution of NtrC, indicating that the kinase activity of NtrB was required. Since NtrB or any other kinase in the $NtrC^{con}$ preparation would have been diluted to the same extent as that added to the wild-type preparation, we conclude that the ATP required for the formation of heparin-resistant complexes is not serving to phosphorylate the activator protein $NtrC^{con}$.

It was necessary to test the second possibility—that ATP conferred heparin-resistance by virtue of allowing transcription to initiate—because we found that ATP could serve as the 5′ nucleotide for glnA transcripts. Using γ-^{32}P–labeled nucleotides, we found that both ATP and GTP (guanosine triphosphate) served as initiating nucleotides for glnA transcripts of *S. typhimurium* (*23*), whereas only ATP served efficiently for those of *Escherichia coli* (*21*). Previous mapping with nuclease S1 localized the start site of the glnA transcript of *Salmonella* to the series of T residues designated as −5 to −1 in Fig. 3 (*9*); on this basis we assigned the A following them as +1. This assignment is also consistent with requirements for synthesis of short abortive transcripts (Fig. 4B) (*24*).

Although ATP could function as the initiating nucleotide at glnA, we showed that this was not its role in conferring heparin resistance, first by showing that there was no correlation between the ability of ATP analogs to confer heparin-resistance and their ability to serve as transcription substrates. The analog 2′,3′-dideoxy ATP, which is not a substrate for transcription, did serve in formation of stable complexes (Fig. 4A, lane 8). In experiments with analogs, ATP was added just after heparin and the remaining three nucleotides to allow stable complexes to synthesize transcripts. The formation of heparin-resistant complexes showed a sigmoidal response to the dideoxy ATP concentration with half-maximal response at approximately 1 m*M* (*21*), an indication that dideoxy ATP served about as well as ATP. Conversely, other analogs such as β,γ-methylene- or β,γ-imido-ATP that serve as substrates for polymerization of glnA transcripts (Fig. 4C, lane 2 and other data) would not serve for stable complex formation (Fig. 4A, lanes 10 and 9). We conclude that the role of ATP in stable complex formation is other than that of the initiating nucleotide (*25*). Although GTP could substitute for ATP (Fig. 4A, lane 4), titration experiments indicated that higher concentrations (about four times as high) were required to obtain equivalent amounts of stable complex formation (*21*).

By substituting dideoxy ATP for ATP we confirmed in two ways that the nucleotide requirement for the formation of heparin-resistant complexes preceded initiation of transcription. First, we deliberately provided all of the nucleotides (or appropriate analogs) necessary for polymerization of full-length glnA transcripts [β,γ-methylene-ATP plus GTP, CTP (cytidine triphosphate), and UTP (uridine triphosphate)] or for polymerization of short abortive transcripts (the dinucleotide ApC plus GTP). This was not sufficient

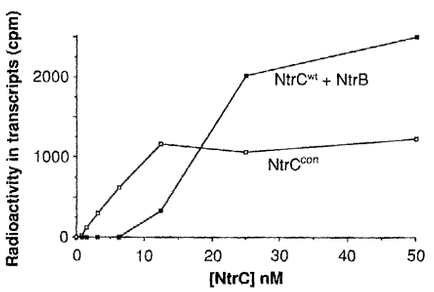

Fig. 2. Dependence of the formation of heparin-resistant complexes on the concentration of $NtrC^{con}$ or wild-type NtrC plus NtrB. Heparin-resistant complexes between σ^{54}-holoenzyme and the *glnA* promoter were formed as described for the "complete" reaction of Fig. 1, except that the concentration of $NtrC^{con}$ (*ntrC610*) or wild-type NtrC (see below) was varied as indicated; heparin-resistant complexes were detected by transcription (see legend to Fig. 1). The ^{32}P incorporated into *glnA* transcripts was quantified by excising the transcripts from gels, solubilizing them overnight in hydrogen peroxide, and counting (Fischer Scintiverse E). The values (counts per minute in transcripts) were corrected for background by subtraction of the average number of counts in gel slices from above and below each transcript band. The final concentrations of wild-type NtrC were obtained by serial dilution of a mixture of wild-type NtrC (1.0 μM) and NtrB (13 n*M*).

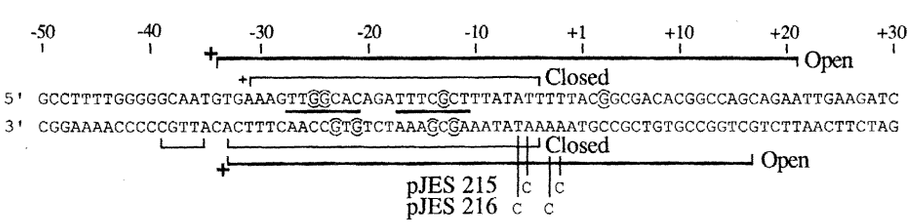

Fig. 3. Summary of protection data for open (heparin-resistant) and closed (heparin-sensitive) complexes between σ^{54}-holoenzyme and the *glnA* promoter. Promoter recognition sequences for σ^{54}-holoenzyme, which are characterized by conserved GG and GC doublets separated by 10 bp (*11*), are indicated by heavy lines between the top and bottom strands. The start site of transcription, +1, was determined from data in (*9*) and Fig. 4 (*24*). Labeled brackets show the extent of protection from DNase I digestion in closed and open complexes (Fig. 5B, top strand). DNase I protection data for the bottom strand is from (*14*) and (*15*) (closed complexes) or (*21*) (open complexes). Ends of the brackets indicate the minimal regions within which protections are observed and are not intended to define the boundaries of protection precisely. Plus signs designate enhancement of DNase I cleavage. Guanine residues protected from methylation by dimethyl sulfate are designated by half circles above and below the base (Fig. 5B, top strand). Position +3 is partially protected only in the open complexes (Fig. 5B). Dimethyl sulfate protection data for the bottom strand in closed complexes is from (*15*) and is similar for open complexes (*21*). Cytosine residues were introduced into the bottom strand at positions −2 and −5 (pJES215) or at −3 and −6 (pJES216) to allow detection of strand separation in open complexes (legend Fig. 6).

to yield heparin-resistant complexes (Fig. 4C, lane 1 and other data) or to allow synthesis of the expected transcripts (Fig. 4C, lane 1, and Fig. 4B, lanes 5 and 4) (*26*); if dideoxy ATP was also provided prior to heparin treatment, heparin-resistance was achieved (Fig. 4C, lane 2) and the expected transcripts were synthesized (Fig. 4C, lane 2, and Fig. 4B, lanes 10 and 9). Since dideoxy ATP cannot be incorporated into transcripts, these results indicate that the requirement for dideoxy ATP, and by inference that for ATP, precedes initiation of transcription. Second, we demonstrated biochemically that providing dideoxy ATP, even at a concentration of 4 m*M*, did not allow synthesis of transcripts; when α-^{32}P–labeled CTP (the nucleotide at position +2) or α-^{32}P–labeled GTP (the nucleotide at positions +3 and +4), or both, were included with the dideoxy ATP during formation of heparin-resistant complexes, neither short abortive transcripts nor longer transcripts were detected (Fig. 4B, lanes 6 to 8). In conjunction with results in Fig. 4A, lane 8, these results indicate that formation of heparin-resistant complexes does not depend on initiation of transcription and demonstrate rigorously that such complexes formed in the presence of dideoxy ATP are pre-initiation complexes. Complexes formed in the presence of 4 m*M* ATP are capable of synthesizing small amounts of 4- and 6-nt transcripts that are released ["abortive" transcripts (*27, 28*); see Fig. 4B, lanes 11 to 13 and (*29*)].

In an earlier study heparin-resistant complexes were formed at the *E. coli* glnA promoter in the presence of ATP, CTP, and GTP because it was incorrectly assumed that UTP was the initiating nucleotide (*18*). We find that heparin-resistant complexes formed under these conditions synthesized long transcripts (>18 nt), as well as large amounts of 3- and 5-nt transcripts (Fig. 4B, lane 15). Thus, the earlier results did not limit the action of NtrC to a pre-initiation step (open complex formation) as was claimed (*18*); they confirmed the conclusion that NtrC was required sometime before completion of short transcripts (*9*). As at the *Salmonella* glnA promoter, heparin-resistant complexes formed at the *E. coli* glnA promoter in the presence of dideoxy ATP could be rigorously defined as pre-initiation complexes (Fig. 4B, lane 14).

Physical characteristics of stable complexes. To determine whether stable heparin-resistant complexes of σ^{54}-holoenzyme at the glnA promoter could be distinguished physically from heparin-sensitive complexes, we subjected them to deoxyribonuclease I (DNase I) (*30*) and dimethyl sulfate (*31*) "footprinting." Since stable complexes could not be formed with 100 percent efficiency on linear templates, we isolated them from preparative nondenaturing gels (Fig. 5A) (*32–34*) in order to obtain unambiguous footprints. Mobility of the template was apparently retarded in stable complexes because requirements for the formation of the retarded species in Fig. 5A, lane 2, were the same as those for formation of heparin-resistant complexes detected transcriptionally (Fig. 1, lanes 1 and 6). If AMP (adenosine monophosphate) was substituted for ATP (Fig. 5A, lane 1) or if NtrC^{con}, σ^{54}, or core polymerase was omitted from the incubation mixture (*21*), no retarded species was observed. Because we used a promoter fragment that lacked high-affinity binding sites for NtrC (legend to Fig. 5A), the NtrC^{con} activator protein did not bind to it stably to produce a retarded species (Fig. 5A, lane 1).

DNase I footprints of stable heparin-resistant complexes extended

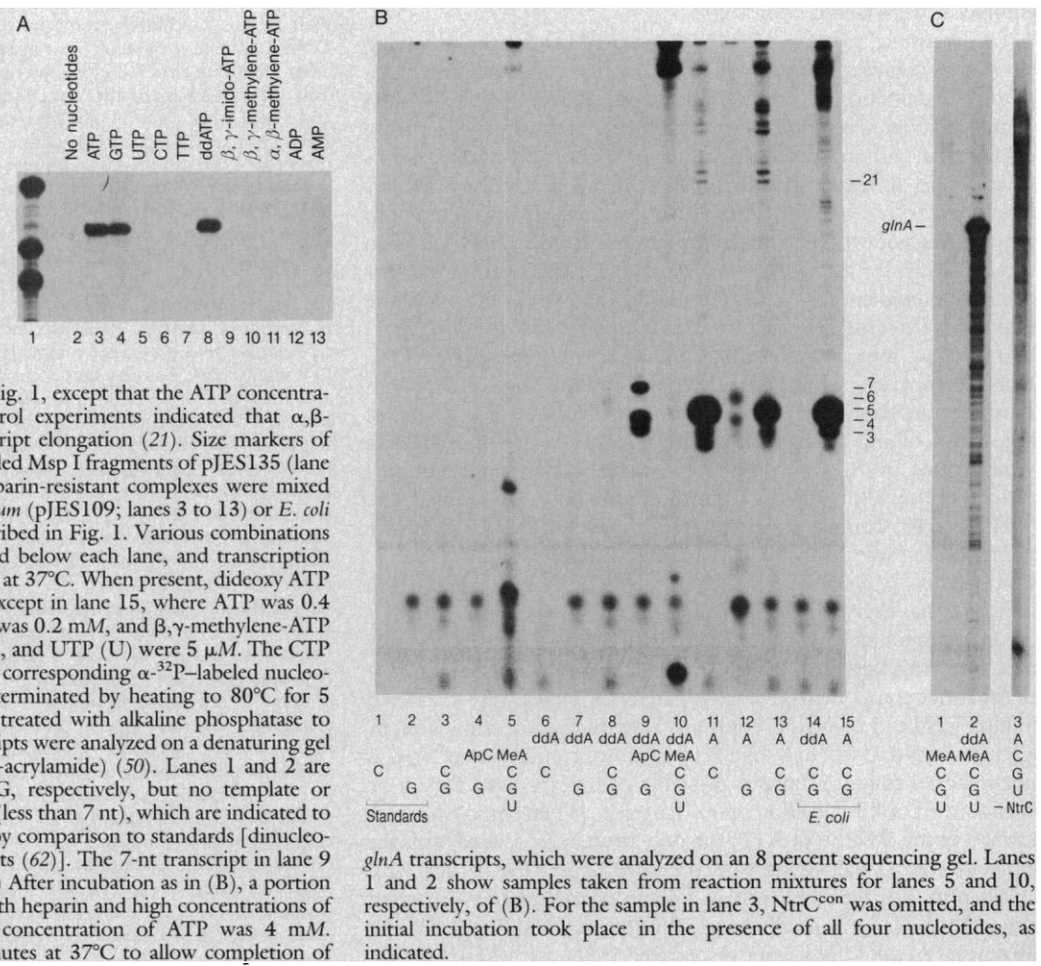

Fig. 4. Use of ATP analogs in formation of heparin-resistant complexes (A and C) and synthesis of transcripts in the presence of various combinations of nucleotides (B). (**A**) Formation of heparin-resistant complexes at the *glnA* promoter was as described for the "complete" reaction of Fig. 1, except that the indicated nucleotides or analogs [10 m*M* final concentration; β,γ-imido-ATP (Serva) and the other nucleotides (Pharmacia)] were substituted for ATP. After the heparin was added, complexes were assayed by allowing transcription as in Fig. 1, except that the ATP concentration was adjusted to 10 m*M*. Control experiments indicated that α,β-methylene-ATP did not inhibit transcript elongation (*21*). Size markers of >620 nt, 527 nt, and 404 nt were labeled Msp I fragments of pJES135 (lane 1). (**B**) Proteins required to form heparin-resistant complexes were mixed with a *glnA* template from *S. typhimurium* (pJES109; lanes 3 to 13) or *E. coli* [pTH8 (*10*); lanes 14 and 15] as described in Fig. 1. Various combinations of nucleotides were added as indicated below each lane, and transcription was allowed to proceed for 15 minutes at 37°C. When present, dideoxy ATP (ddA) and ATP (A) were at 4 m*M* [except in lane 15, where ATP was 0.4 m*M* as in (*18*)], the dinucleotide ApC was 0.2 m*M*, and β,γ-methylene-ATP (MeA) was 1 m*M*; CTP (C), GTP (G), and UTP (U) were 5 μ*M*. The CTP and GTP each contained 5 μCi of the corresponding α-^{32}P–labeled nucleotide per reaction. Transcription was terminated by heating to 80°C for 5 minutes, and reaction mixtures were treated with alkaline phosphatase to remove 5′ triphosphates. Short transcripts were analyzed on a denaturing gel (20 percent acrylamide, 3 percent bis-acrylamide) (*50*). Lanes 1 and 2 are controls that contained only C or G, respectively, but no template or proteins. Sizes of the short transcripts (less than 7 nt), which are indicated to the right of the gel, were determined by comparison to standards [dinucleotide-initiated 3-, 4-, and 6-nt transcripts (*62*)]. The 7-nt transcript in lane 9 probably ends in ddA (see Fig. 3). (**C**) After incubation as in (B), a portion of each reaction mixture was mixed with heparin and high concentrations of nucleotides as in Fig. 1. The final concentration of ATP was 4 m*M*. Incubation was continued for 10 minutes at 37°C to allow completion of *glnA* transcripts, which were analyzed on an 8 percent sequencing gel. Lanes 1 and 2 show samples taken from reaction mixtures for lanes 5 and 10, respectively, of (B). For the sample in lane 3, NtrC^{con} was omitted, and the initial incubation took place in the presence of all four nucleotides, as indicated.

approximately 20 base pairs (bp) past the transcription start site (Fig. 5B, lane 3), and thus were readily distinguished from the shorter footprints of unstable heparin-sensitive complexes (Fig. 5B, lane 2) (*14, 15*). [Footprints of stable complexes formed in the presence of ATP or dideoxy ATP were the same (*21*).] Differences in methylation protections between stable and unstable complexes were subtle: the G residue at position +3 was protected only in the stable complexes; the G's at positions −13, −24, and −25 within the promoter were protected in both types of complexes but that at position −13 appeared to be somewhat less well protected in the stable complexes (Fig. 5B, lanes 10 and 11).

To demonstrate that stable heparin-resistant complexes between σ^{54}-holoenzyme and the glnA promoter were open complexes, we detected single-stranded cytosines between the promoter and the transcription start site. Wang and his colleagues (*35*) demonstrated that the N-3 position of cytosine was sensitive to methylation in single-stranded but not duplex DNA, and used this sensitivity to demonstrate localized strand denaturation in open complexes formed by σ^{70}-holoenzyme, the most abundant holoenzyme form of RNA polymerase in enteric bacteria. To study cytosine methylation at the *Salmonella* glnA promoter, we used synthetic oligonucleotides to introduce C residues into the template (bottom) strand between the promoter recognition sequence at −14 and the transcription start site (see legend to Fig. 6). In heparin-resistant complexes formed with either ATP or dideoxy ATP (equivalent to retarded species in Fig. 5A), these cytosines were methylated (Fig. 6, lanes 1 and 3), an indication that they were unpaired and hence that stable heparin-resistant complexes were open complexes. The cytosines were not methylated in heparin-sensitive complexes formed in the presence of AMP (Fig. 6, lanes 2 and 4), an indication that such complexes were closed complexes.

To determine whether NtrC was required for events occurring after open complex formation—that is, maintenance of open complexes or initiation of transcription—we separated stable open complexes from free proteins on a high-resolution molecular sizing column (*34, 36*) and determined their protein composition. Complexes were formed on a plasmid template that lacked specific binding sites for NtrC; any $NtrC^{con}$ nonspecifically associated with the template or weakly associated with σ^{54}-holoenzyme was then titrated away by addition of an oligonucleotide bearing a high-affinity binding site for NtrC. This 21-bp oligonucleotide had little inhibitory effect on transcription if added after stable complexes had been formed, but it was strongly inhibitory if added earlier (*21*). Stable complexes, which were detected on the basis of heparin-resistant transcriptional activity, were eluted in the void volume of the sizing column along with the template peak. When complexes were formed in the absence of ATP, there was no transcriptional activity in the void volume of the column (*21*).

The protein composition of stable complexes isolated from the sizing column was determined by immunoblotting (Fig. 7) (*36, 37*). Comparison to protein standards (Fig. 7, lanes 1 to 11 and 15) indicated that they contained σ^{54} and the subunits of core polymerase in approximately equimolar amounts (Fig. 7, lane 13) but that they contained very little NtrC (less than 15 percent of the amount of the other components). Since isolated complexes were recovered in high yield and their transcriptional activity was not stimulated by addition of $NtrC^{con}$, NtrB, and ATP (*21*), we conclude that NtrC is not required for maintenance of stable complexes once they have been formed or for initiation of transcription. When complexes were formed in the absence of ATP, the only protein associated with the template was a small amount of core polymerase (Fig. 7, lane 12).

When we isolated stable initiated complexes that had synthesized either 21- or 22-nt transcripts and 28-nt transcripts (70 percent and 30 percent of total transcripts, respectively) (*21*), we found that they

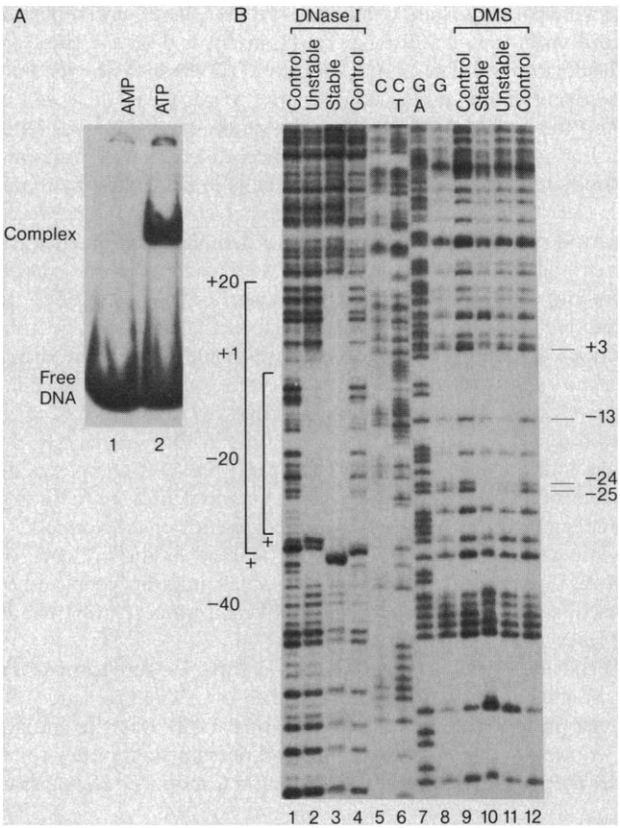

Fig. 5. Isolation of stable (heparin-resistant) complexes (A) and analysis of their DNase I and methylation protection patterns (B). (**A**) An end-labeled DNA fragment (~3 n*M*) bearing the *glnA* promoter, but lacking high affinity binding sites for NtrC, was incubated for 20 minutes at 37°C with 160 n*M* core RNA polymerase, 192 n*M* σ^{54}, 200 n*M* $NtrC^{con}$, 17 n*M* NtrB, and 4 m*M* AMP (lane 1) or ATP (lane 2); the buffer was as described in the legend to Fig. 1 and the total volume was 30 μl. (NtrB was added to increase the yield of heparin-resistant complexes.) Heparin (to 50 μg/ml) and sucrose (to 6 percent) were added, and the mixtures were subjected to electrophoresis at 15 V/cm for 2 hours at 30°C in a 5 percent nondenaturing polyacrylamide gel (*32, 33*) equilibrated in 20 m*M* tris-HCl, 2 m*M* EDTA, *p*H 8.0. The gel was exposed to Kodak XAR film. The 380 bp Hind III–Bst NI fragment of pJES124 was labeled as described (*60*) at the Hind III site on the nontemplate (top) strand at a position 110 bp upstream of the transcription start site. pJES124 contains a Bgl II–Hph I fragment of the *S. typhimurium glnA* promoter region [positions −100 to +30, including the low affinity NtrC binding sites 3, 4, and 5 (*9*)] inserted between the Bam HI and Pst I sites of pTE103 (*61*). (**B**) Unstable, heparin-sensitive complexes formed in the presence of AMP [as for (A), lane 1] or free *glnA* promoter fragment were treated with DNase I or dimethyl sulfate essentially as described (*15*). Lanes 1 and 2 show DNase I digestion patterns of the *glnA* promoter fragment in the absence of proteins (control; lane 1) or in unstable complexes (lane 2) (*14, 15*). Similarly, lanes 11 and 12 show methylation patterns in unstable complexes (lane 11) or the control (lane 12) (*15*). Lanes 3 and 4, respectively, show DNase I digestion patterns for stable complexes formed as in lane 2 (A), or free promoter fragment (both isolated from a nondenaturing gel). Similarly, lanes 10 and 9 show methylation patterns for stable complexes (lane 10) or free promoter fragment (control; lane 9). Lanes 5 to 8 show chemical sequencing reactions for the *glnA* promoter fragment (60). For methylation experiments Hepes replaced tris as the buffering agent during formation of complexes (*35*). Just before electrophoresis on nondenaturing gels, stable complexes or free fragment were subjected to DNase I digestion (0.8 ng/ml; Calbiochem) (*30, 33*) for 30 s or to methylation by dimethyl sulfate (35 m*M*; Aldrich) (*31*) for 4 minutes, both at 37°C. Heparin and sucrose were added [as in (A)] and samples were placed on a nondenaturing gel that was already running (*33*). After electrophoresis and autoradiography of the gel, the appropriate bands were excised, electroeluted, and precipitated with ethanol. Methylated promoter fragments were cleaved in 1*M* piperidine for 30 minutes at 90°C and lyophilized (*31*). All samples were then lyophilized twice from water, resuspended in 80 percent formamide, and analyzed on a 10 percent sequencing gel (*60*).

contained no detectable σ^{54} (Fig. 7, lane 14); only the subunits of core polymerase were present. This result is consistent with the view that σ^{54} dissociates from core during synthesis of 28-nt transcripts; alternatively, it could have dissociated during chromatography. The result further indicates that all molecules of σ^{54}-holoenzyme that are capable of forming stable open complexes are also able to initiate and elongate transcripts.

Roles of NtrC and ATP in open complex formation. In the presence of ATP or dideoxy ATP, the NtrC activator protein catalyzes isomerization of closed recognition complexes between σ^{54}-holoenzyme and the glnA promoter to open complexes. Sasse-Dwight and Gralla recently demonstrated that NtrC is required for this isomerization in vivo (*38*). Closed complexes (*20, 27, 39*) were defined as such by the following criteria. (i) They were readily detectable physically by DNase I and methylation protection assays (Fig. 5) (*14, 15*) but the DNA strands did not appear to be denatured (Fig. 6). (ii) They were nonproductive in transcription even in the presence of all four nucleotides (*9, 15*) (Fig. 4C, lane 3). Because these complexes can be studied at physiological temperatures (37°C) (*40*), they are almost certainly on the pathway to open complex formation. In heparin-resistant, open complexes (*20, 27, 39*), which are productive transcriptionally, we showed directly that the DNA strands were denatured between the promoter and the glnA transcription start site (Fig. 6); in addition, the DNase I footprint of open complexes was longer than that of closed complexes, extending to approximately 20 bp downstream of the transcription start site (Figs. 3 and 5). Physical changes upon the progression from closed to open complexes indicated that σ^{54}-holoenzyme had undergone a conformational change with respect to the template.

Like other sigma factors, σ^{54} confers on core RNA polymerase the ability to bind specifically to a promoter—a minimal definition of a sigma factor (*12*)—but it apparently does not confer the ability to form open complexes even at 37°C (*40, 41*). In this regard, it is interesting that σ^{54} (*21, 42, 43*) shows little or no sequence similarity to the other known sigma factors (*13, 43*), of which approximately 15 have now been identified; these, which share several regions of conserved amino acid sequence (*13, 44*), do permit open complex formation at least at some promoters. The separation of functions between σ^{54} and the activator NtrC is consistent with the view that other sigma factors are responsible for localized denaturation of the DNA strands as well as for promoter recognition (*13*). Once open complexes have been formed, NtrC is not required to maintain them or to initiate transcription (Fig. 7)—σ^{54}-holoenzyme by itself is capable of these functions. Thus the role of NtrC is localized to formation of open complexes.

The ATP required for the formation of open (heparin-resistant) complexes between σ^{54}-holoenzyme and the glnA promoter was required prior to initiation of transcription (Fig. 4) and was not required simply to phosphorylate NtrC (Fig. 2) (*45*). Although we do not know the precise role of ATP in open complex formation, we think that it is used by NtrC rather than by σ^{54}-holoenzyme because a putative ATP binding site has been identified in the central domain of NtrC and other activators of σ^{54}-holoenzyme (*46*). This view is also consistent with the fact that formation of open complexes at the glnA promoter showed a sigmoidal dependence on ATP concentration (*21*), because NtrC exists as a dimer in solution

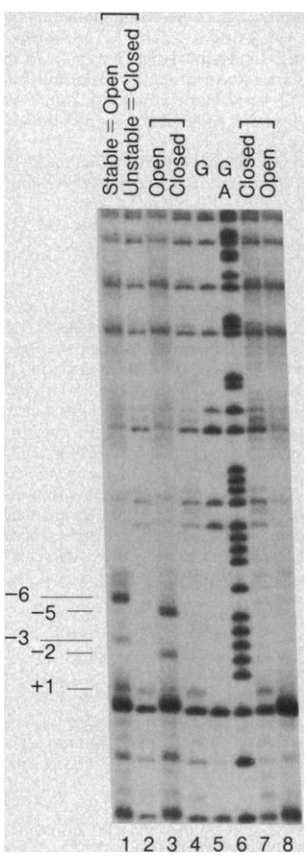

Fig. 6. Methylation of the N-3 position of cytosine to assess strand separation in stable complexes. Unstable and stable complexes between σ^{54}-holoenzyme and the *glnA* promoter were formed in the presence of AMP (lanes 2, 4, and 7; unstable indicated by "closed") or ATP, respectively (lanes 1, 3, and 8; stable indicated by "open"), as described in the legend to Fig. 5A. Treatment with dimethyl sulfate and isolation of stable, heparin-resistant complexes from nondenaturing gels was as described in the legend to Fig. 5B. In this experiment unstable complexes were also subjected to nondenaturing electrophoresis and the "free DNA" which they yielded (Fig. 5A, lane 1) was excised for further analysis. After they were precipitated with ethanol, the DNA fragments were treated with hydrazine and chemically cleaved at N^3-methylcytosines (and N^7-methylguanines) by the method of Kirkegaard *et al* (*35*). Promoter fragments were derived from pJES214 (lanes 7 and 8), which contains the wild-type *glnA* promoter from positions −32 to +17 (Fig. 3), but lacks binding sites for NtrC (*9, 15*) or from pJES216 (lanes 1 and 2), in which positions −6 and −3 have been changed to G · C pairs (Fig. 3) or from pJES215 (lanes 3 and 4), in which positions −5 and −2 have been changed to G · C pairs (Fig. 3). All three plasmids were constructed by ligating complementary 55-base oligonucleotides with appropriate cohesive ends between the Eco RI and Bam HI sites of the vector pTE103 (*61*). The 285-bp Hind III–Bst NI fragments were ^{32}P-labeled at the Hind III site on the template (bottom) strand at a position corresponding to +42. Chemical sequencing of the wild-type promoter from pJES214 (lanes 5 and 6) and electrophoresis were as described in the legend to Fig. 5B.

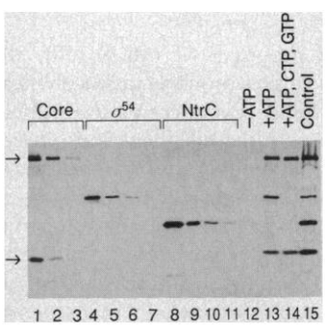

Fig. 7. Protein composition of open (stable, heparin-resistant) complexes and initiated complexes. The *glnA* template [pJES107, which lacks binding sites for NtrC (*15*)], and proteins required for open complex formation were mixed at high concentration in transcription buffer (pJES107, core polymerase, and σ^{54} were present at 166 n*M*; NtrC^{con} at 440 n*M* and NtrB at 17 n*M*) and were then incubated for 20 minutes at 37°C with 4 m*M* ATP (lane 13) or without ATP (lane 12). After the initial incubation, a synthetic 21-bp DNA fragment bearing a high-affinity binding site for NtrC (12.5 μ*M*) was added, and incubation was continued for 5 minutes. The mixtures (100 μl) were then chilled to 4°C and fractionated by molecular size on a Superose 12 column (25 ml; Pharmacia) in transcription buffer without polyethylene glycol. If complexes were formed in the presence of ATP, a peak of heparin-resistant transcriptional activity coincided with the template peak, which eluted near the void volume of the column and was detected by absorbance at 280 nm. Recovery of transcriptional activity was quantitative; [comparison of the activity in column fractions and starting material corrected for losses as a result of decay (*34*)]. To form initiated complexes (lane 14) initial incubation was in the presence of ATP, and CTP and GTP (10 μ*M*) were added at the same time as the synthetic NtrC binding site. Samples (250 μl) of fractions containing the template peak for each experiment were concentrated by precipitation with trichloroacetic acid (7 percent final concentration), washed with acetone, subjected to polyacrylamide gel electrophoresis, and analyzed by immunoblotting (*36, 37*) with a mixture of rabbit polyclonal antisera to core polymerase (*62*), σ^{54}, and NtrC. To assess recovery in the concentration step, an equimolar mixture of core, σ^{54}, and NtrC was concentrated in parallel with other fractions (control; lane 15). Standards contained 0.33, 0.08, and 0.02 pmole of core RNA polymerase (lanes 1 to 3) or 0.67, 0.17, 0.04, and 0.01 pmole, respectively, of σ^{54} (lanes 4 to 7) or NtrC (lanes 8 to 11). Concentrations were calculated from measurements of absorbance at 280 nm with the use of the extinction coefficients of the proteins. The β, β′ subunits and the α subunit of core are designated by the upper and lower arrows, respectively.

(*21, 47*). ATP analogs with an altered triphosphate moiety did not permit open complex formation (Fig. 4A, lanes 9 to 13), suggesting that ATP hydrolysis may be necessary.

It has been proposed that the MalT (maltoseT) activator protein of *E. coli* may require ATP to stimulate initiation of transcription by σ^{70}-holoenzyme from maltose promoters (*48*). ATP is required before or concomitant with synthesis of the first phosphodiester bond by eukaryotic RNA polymerase II (*49, 50*).

Domain structure of NtrC and enhancer binding. The activator protein NtrC, 54 kD, appears to comprise three functional regions (*51*). (i) The large central domain, approximately 26 kD, which includes the ATP binding site discussed above (*46*), is probably directly responsible for stimulating open complex formation by σ^{54}-holoenzyme because it is conserved among the known activators for this form of RNA polymerase [NtrC (*51–53*), NifA (*51, 52*), and DctD (*46*); there is no sequence available for XylR] (*54*). Consistent with this assignment, two point mutations within the central domain of NtrC resulted in loss of ability to activate transcription by σ^{54}-holoenzyme but did not affect functions assigned to other domains (*21, 55*). (ii) The NH_2-terminal domain of NtrC, approximately 13 kD, has regulatory function and appears to control the ability of the central domain to activate transcription by σ^{54}-holoenzyme (*17*). Wild-type NtrC is made in an inactive (unphosphorylated) form, and its activity is regulated positively by phosphorylation and negatively by dephosphorylation (*16*) of its NH_2-terminal domain (*17*). The degree of phosphorylation of NtrC is controlled by the nitrogen status of the cell (*15–17*). (iii) The COOH-terminal region of NtrC is responsible for specific DNA binding: Mutant NtrC proteins defective in DNA binding ability have amino acid changes in the turn or recognition helix of a bihelical DNA binding motif (*56*) that is located five amino acids from the COOH-terminus (*51*).

In agreement with results and proposals of others (*57*), we envision that NtrC bound to enhancer sites probably makes physical contact with σ^{54}-holoenzyme at the glnA promoter to cause isomerization of closed to open complexes. Although there is no direct evidence for such contact, the fact that binding sites for NtrC must be a certain minimum distance from the promoter to function—at least 70 bp—is consistent with this view (*2, 58*); the minimum distance would presumably be set by a requirement to alter the conformation of the intervening DNA (by bending, kinking, or looping) so that bound proteins could interact. In this scheme, binding to enhancer-like sites would serve to raise the local concentration of NtrC (*59*) and to orient NtrC molecules favorably with respect to σ^{54}-holoenzyme bound at the promoter. Interestingly, both in vivo (*2*) and in vitro (*15, 18*, and data shown above) NtrC can activate transcription with low efficiency from templates that lack specific binding sites for it. There are two lines of evidence that it must bind to such templates to activate (*21, 55, 56*): (i) A 21-bp oligonucleotide carrying a high-affinity binding site for NtrC or larger fragments or plasmids carrying such sites are highly inhibitory to formation of heparin-resistant complexes on a template that lacks specific binding sites. (ii) Relative to wild-type NtrC, a mutant NtrC protein that has decreased affinity for DNA due to a change in its DNA binding motif has greatly decreased ability to activate transcription from templates lacking specific binding sites as well as from templates that have them.

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23. Further experiments demonstrated that the ratio of A and G initiated transcripts was proportional to the relative concentrations of ATP and GTP (*21*); thus in the experiments shown in Figs. 1, 2, and 4, transcripts would have initiated predominantly with ATP.
24. Other possibilities for the start site are the A's at positions −6 and +7 of Fig. 3, both of which are less consistent with results of S1 mapping experiments (*9*). Use of the dinucleotide ApC with GTP for synthesis of trimers, tetramers, and heptamers in the presence of dideoxy ATP (Fig. 4) confirms that the start is at +1, as does synthesis of 21-, 22-, and 28-nt transcripts from the substrates ATP, CTP, and GTP (*21*).
25. Further support for the view that heparin-resistance does not require initiation of transcription comes from studies of the *nifLA* promoter of *K. pneumoniae*, transcription from which is also dependent on σ^{54}-holoenzyme and NtrC (*6*). As at *glnA*, either ATP or dideoxy ATP stimulates the formation of heparin-resistant complexes; however, experiments with γ-^{32}P–labeled nucleotides indicate that transcripts initiate only with G, and the first A residue does not occur until position +11 (*21*).
26. Transcripts at the top of the gel in Fig. 4B, lane 5, did not originate from the *glnA* promoter; when completed (Fig. 4C, lane 1) they migrated more slowly than full-length *glnA* transcripts (Fig. 4C, lane 2).
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41. The *glnA* promoter, the site of our study of the function of σ^{54}-holoenzyme, is a strong promoter in vivo. From it glutamine synthetase can be expressed to several percent of cellular protein under appropriate metabolic conditions (nitrogen limiting) (*14*). The σ^{54}-holoenzyme requires an activator to form open complexes not only at *glnA* but also at more than a dozen other promoters; moreover, there is no counter example of a promoter from which it can transcribe in the absence of an activator. Together these observations suggest that this form of RNA polymerase is not capable of isomerizing to an open complex by itself.
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