Converting *Escherichia coli* RNA Polymerase into an Enhancer-Responsive Enzyme: Role of an NH₂-Terminal Leucine Patch in σ₅⁴

Jonathan T. Wang, Adeela Syed, Mingli Hsieh,* Jay D. Gralla†

The protein σ₅⁴ associates with *Escherichia coli* core RNA polymerase to form a holoenzyme that binds promoters but is inactive in the absence of enhancer activation. Here, mutants of σ₅⁴ enabled polymerases to transcribe without enhancer protein and adenylate triphosphate. The mutations are in leucines within the NH₂-terminal glutamine-rich domain of σ₅⁴. Multiple leucine substitutions mimicked the effect of enhancer protein, which suggests that the enhancer protein functions to disrupt a leucine patch. The results indicate that σ₅⁴ acts both as an inhibitor of polymerase activity and as a receptor that interacts with enhancer protein to overcome this inhibition, and that these two activities jointly confer enhancer responsiveness.

Enhancer-dependent transcription in *E. coli* requires the alternative σ factor σ₅⁴ (1). This protein associates with the common core RNA polymerase used by all σ factors, including σ₇₃; σ₅⁴ association makes polymerase responsive to a number of diverse enhancer-binding proteins. Although enhancer-dependent transcription is unusual in bacteria, it is common in eukaryotes, and the simple σ₅⁴ system appears to be a hybrid between eukaryotic and prokaryotic mechanisms (2). How σ₅⁴ alters bacterial polymerase to convert it to an enhancer-responsive form is unknown. Here, we addressed this question by identifying mutants of σ₅⁴ that allowed the polymerase to transcribe independently of an enhancer protein. We chose this approach because the localization of such mutations would define the σ₅⁴ domains responsible for conferring enhancer responsiveness to the polymerase, and because we felt that the properties of the mutated σ₅⁴ holoenzyme would provide mechanistic insight about enhancer-dependent transcription.

Our previous studies of a large number of σ₅⁴ mutants, including point mutations and deletions (3–5), suggested that σ₅⁴ has an activation domain near the NH₂-terminus, which contains many interdigitated leucine and glutamine residues. Here, we screened these mutants for σ₅⁴ promoter-driven gene expression that was enhancer protein independent. Twenty plasmids with different mutations in the leucine and glutamine motifs of σ₅⁴ were transformed into *E. coli* strain YMC12 (6), which lacks enhancer protein NtrC (also known as GlgG and NRI). These bacteria were plated on glucose-glutamine plates (3); large colonies did not form on such plates because wild-type σ₅⁴ cannot contribute to the transcription of genes necessary for glutamine transport and synthesis without NtrC. Of the 20 NH₂-terminal mutants, three showed positive growth (Fig. 1), which indicated that they contained forms of σ₅⁴ that could direct transcription without the activator NtrC. Several mutants in other regions of σ₅⁴ were also tested, but none showed growth comparable to that of the three NH₂-terminal mutants (7).

All three mutants that could grow without NtrC had changes in leucine residues (Fig. 1). In addition, three other mutants showed weaker but detectable growth, and these mutations also affected leucines. All six mutants that showed growth had changes in a small subregion between Leu25 and Leu37. Changes in glutamines, however, did not result in growth, although some mutants had glutamine changes in the same subregion. We infer that changes in leucine residues, particularly those in this subregion, are important for conferring an enhancer-independent phenotype.

Three forms of mutant σ₅⁴ protein were purified for further study in vitro, along with wild-type protein (8). Two of these three proteins were the presumptive enhancer-independent leucine mutants LS2633 and HRS456. The other was QS3, which had changes in glutamines within the same subregion as the leucine mutants but was not an enhancer-independent mutant. In vitro transcription (9) showed that the two leucine mutants could transcribe from the glnAp2 promoter (10) in vitro in the absence of enhancer protein NtrC (Fig. 2). By contrast, neither the wild type nor the glutamine mutant QS3 could transcribe in vitro without NtrC.

The requirements for enhancer-independent transcription were delineated with LS2633. As a control, transcription with wild-type σ₅⁴ holoenzyme was shown to require phosphorylated NtrC and adenylate triphosphate (ATP) (Fig. 3). Transcription with LS2633 holoenzyme required neither enhancer protein nor the presence of a β-γ

![Fig. 2](http://science.sciencemag.org/)

**Fig. 2.** In vitro transcription with the use of mutant σ₅⁴ proteins. The indicated forms of σ₅⁴ were used in reactions with or without NtrC.

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Notes:

- Department of Chemistry and Biochemistry and Molecular Biology Institute, University of California, Los Angeles, CA 90095, USA.
- *Present address: Institute of Medicine, Chung Shan Medical and Dental College, Taichung, Taiwan.
- †To whom correspondence should be addressed.
Wild type       LS2633
NtrC  +  ATP  AMPPPNP

Fig. 3. Requirements for transcription by enhancer-independent mutant LS2633. Components omitted from the standard complete system are indicated and the results are compared for LS2633 and wild-type α proteins. CP, carbamyl phosphate.

hydrolyzable form of ATP (Fig. 3) (11). The minimal requirements for LS2633 transcription included mutant α54, polymerase, and elongation substrates [lane 14 of Fig. 3 uses the β-γ nonhydrolyzable ATP analog adenyl imidodiphosphate (AMPNP)]. Typical prokaryotic transcription does not require ATP β-γ bond hydrolysis, whereas enhancer-dependent transcription, both in prokaryotes and eukaryotes, requires ATP hydrolysis (12). This result confirms the suggestion that ATP hydrolysis (13) in the α54 system is a necessary activity of the enhancer protein rather than a part of the holoenzyme; in instances in which the enhancer was not required, neither was ATP.

LS2633 began transcription without a discernible lag, consistent with the lack of a need to accommodate the slow enhancer-dependent steps (Fig. 4) (14). In contrast, wild-type transcription occurred only after a delay, which presumably involved the phosphorylation of NtrC, its binding to DNA, and its looping to touch the polymerase (10, 13, 15). Thus, the LS2633 mutation of α54 causes the polymerase to behave like α70 forms of the holoenzyme, because it binds to a promoter in a transcriptionally active form (Figs. 3 and 4) (16).

The activation event caused by the enhancer protein and ATP in the case of wild-type α54 is the formation of a heparin-resistant (10, 17) open complex at the glnAp2 promoter. Heparin-resistant glnAp2 transcription required NtrC for wild-type α54 (Fig. 5). Thus, we investigated whether LS2633 could direct heparin-resistant transcription in the absence of NtrC. The results showed that LS2633 could only do so under specialized conditions. LS2633 transcription was sensitive to heparin (Fig. 5, lanes 7 and 8); however, heparin-resistant transcription was observed when the LS2633 transcription complex was preincubated with nucleotides, which allowed the formation of more than one phosphodiester bond (Fig. 5, lanes 9 and 10). In this respect, LS2633 differs from wild-type α54, which could not form heparin-resistant complexes in the absence of NtrC, even when nucleotides were present (Fig. 5).

These results imply that although the LS2633 holoenzyme can form heparin-resistant complexes in the absence of NtrC, this process requires the polymerase to use nucleotides to form a short RNA, six nucleotides in this case. When a six-nucleotide RNA forms at the glnAp2 promoter, the polymerase has just entered an elongation mode (18). Before initiation with nucleotides, the mutant transcription complexes appear to include an equilibrium mixture of open and closed complexes; the presence of the closed complexes accounts for the inactivation by heparin. This view is supported by permanganate footprinting (19) experiments (Fig. 6). The two mutant proteins LS2633 and HS456 yielded detectable open-complex signals, which were further enhanced by the presence of NtrC. Quantification of several experiments with the mutants indicated that ~15% of the promoter DNA is in the open-complex state in the absence of NtrC, compared to less than 1% opened by the wild-type α. Taken together with the results in Fig. 5, these data indicate that the mutations in α54 cause the polymerase to open the DNA long enough to direct the condensation of nucleotides and transcription initiation. This 15% opening drives much more than 15% transcription (Fig. 4); as the open complexes are depleted by initiation, the disturbed equilibrium should drive the formation of new open complexes that continue to initiate.

Our results provide clues about how α54 confers enhancer responsiveness to E. coli RNA polymerase. The NH2-terminus of the protein contains a leucine-rich region that holds the activity of core polymerase in check. When this leucine patch is mutated, the polymerase can transcribe even without enhancer protein. Thus, the leucine patch functions to change the bound polymerase to keep it from opening the DNA and transcribing. A key property of the α54 holoenzyme is the unusual ability to form an inactive stable closed complex (2). By contrast, α70 lacks this ability, perhaps because it lacks a motif that holds the DNA-melting function of the holoenzyme in check.

Because the leucine disruption mutations of α54 mimic the activation function of NtrC, we infer that a likely role of NtrC is to disrupt interactions involving this leucine patch. In this view, the hydrophobic leucines participate in interactions (disrupted by changing to hydrophilic serines) that keep the start site closed, and NtrC disrupts these interactions. Thus, the role of the enhancer is to counteract a leucine patch-dependent inhibition of DNA-melting activity. Although the interdigitated glutamines are apparently not involved in keeping this melting in check, they are required for the positive response to NtrC, as indicated in prior studies (5). Thus, NtrC may act as an enhancer protein by making use of contacts to a glutamine-rich domain to trigger a change in the structure of the region. In this model, two key features of the NH2-terminus of α54 act together to confer enhancer responsiveness on polymerase: (i) features that prevent transcription without activation (leucines in this case), and (ii) features that allow an enhancer protein to overcome this repression (likely including glutamines). Because both leucine and glutamine motifs also exist in proteins that are involved in eukaryotic transcription, the model may apply to enhancer-dependent eukaryotic transcription.

REFERENCES AND NOTES

Transition in Specification of Embryonic Metazoan DNA Replication Origins

Olivier Hyrien,† Chrystelle Maric,† Marcel Méchali*

In early Xenopus embryos, in which ribosomal RNA genes (rDNA) are not transcribed, rDNA replication initiates and terminates at 9- to 12-kilobase pair intervals, with no detectable dependency on specific DNA sequences. Resumption of ribosomal RNA (rRNA) synthesis at late blastula and early gastrula is accompanied by a specific repression of replication initiation within transcription units; the frequency of initiation within intergenic spacers remains as high as in early blastula. These results demonstrate that for rRNA genes, circumscribed zones of replication initiation emerge in intergenic DNA during the time in metazoan development when the chromatin is remodelled to allow transcription.

Eukaryotic DNA replication initiates at multiple replication origins spread along the length of each chromosome. In yeast, replication origins correspond to specific nucleotide sequences (1). In higher eukaryotes, the nature of replication origins is much less clear. For example, initiation can occur at any of a large number of sites in a 55-kbp zone downstream of the Chinese hamster dhfr gene (2). In metazoan cells (1, 2). On the other hand, replication of the human β-globin gene cluster may be regulated by a DNA sequence located between the 8- and 9-globin genes (5).

The tandemly repeated, highly conserved rRNA genes (Fig. 1A) provide an interesting model of eukaryotic replicons. Electron microscopy studies of replicating DNA or chromatin have long suggested that initiation is restricted to the rDNA intergenic spacer in many protozoan and metazoan species [References in (6, 7)]. Analysis of replication intermediates (RIs) by two-dimensional (2D) gel electrophoresis confirmed that replication initiates in the rDNA spacer, the noncoding sequences between genes, at specific sites in Saccharomyces cerevisiae (8, 9) and Physarum polycephalum (6) and in a broad zone in human cells (10). A replication fork barrier was also found at the 3' end of ribosomal genes in several species (7–11).

In contrast to this conserved pattern of fixed sites or zones of initiation and termination, replication initiates and terminates at random sequences, though at regular 9- to 12-kilobase pair intervals, in the rDNA of Xenopus early blastulae (12). Plasminogenactivating softăm→s,

Molecular Embryology, Institut Jacques Monod, 75 251 Paris Cedex 05, France.

†To whom correspondence should be addressed.

*Present address: Génétique Moléculaire, Département de Biologie, Ecole Normale Supérieure, 46 rue d'Ulrm, 75230 Paris Cedex 05, France.
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