Converting *Escherichia coli* RNA Polymerase into an Enhancer-Responsive Enzyme: Role of an NH_2-Terminal Leucine Patch in σ^{54}

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

The protein σ^{54} 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 σ^{54} enabled polymerases to transcribe without enhancer protein and adenosine triphosphate. The mutations are in leucines within the NH_2-terminal glutamine-rich domain of σ^{54}. 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 σ^{54} 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 σ^{54} (*1*). This protein associates with the common core RNA polymerase used by all σ factors, including σ^{70}; σ^{54} 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 σ^{54} system appears to be a hybrid between eukaryotic and prokaryotic mechanisms (*2*). How σ^{54} alters bacterial polymerase to convert it to an enhancer-responsive form is unknown. Here, we addressed this question by identifying mutants of σ^{54} that allowed the polymerase to transcribe independently of an enhancer protein. We chose this approach because the localization of such mutations would define the σ^{54} domains responsible for conferring enhancer responsiveness to the polymerase, and because we felt that the properties of the mutated σ^{54} holoenzyme would provide mechanistic insight about enhancer-dependent transcription.

Our previous studies of a large number of σ^{54} mutants, including point mutations and deletions (*3–5*), suggested that σ^{54} has an activation domain near the NH_2-terminus, which contains many interdigitated leucine and glutamine residues. Here, we screened these mutants for σ^{54} promoter-driven gene expression that was enhancer protein independent. Twenty plasmids with different mutations in the leucine and glutamine motifs of σ^{54} were transformed into *E. coli* strain YMC12 (*6*), which lacks enhancer protein NtrC (also known as GlnG and NRI). These bacteria were plated on glucose-glutamine plates (*3*); large colonies do not form on such plates because wild-type σ^{54} cannot contribute to the transcription of genes necessary for glutamine transport and synthesis without NtrC. Of the 20 NH_2-terminal mutants, three showed positive growth (Fig. 1), which indicated that they contained forms of σ^{54} that could direct transcription without the activator NtrC. Several mutants in other regions of σ^{54} were also tested, but none showed growth comparable to that of the three NH_2-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 Leu^{25} and Leu^{37}. 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.

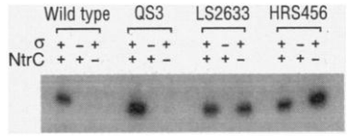

Fig. 2. In vitro transcription with the use of mutant σ^{54} proteins. The indicated forms of σ^{54} were used in reactions with or without NtrC.

Three forms of mutant σ^{54} 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 σ^{54} holoenzyme was shown to require phosphorylated NtrC and adenosine triphosphate (ATP) (Fig. 3). Transcription with LS2633 holoenzyme required neither enhancer protein nor the presence of a β-γ

		1 . . . 10 . . . 20 . . . 30 . . . 40
Wild type	−	M K Q G L Q L R L S Q Q L A M T P Q L Q Q A I R L L Q L S T L E L Q Q E L Q Q A
LS2633	+	S S
HRS456	+	S S S
HRS1256	+	S S S S
HRS56	+/−	S S
HRS4	+/−	S
HRS5	+/−	S
HRS2	−	S
LS19	−	S
LS33	−	S
HRS1	−	S
HRS12	−	S S
HRS1-3	−	S S S
HRS1-4	−	S S S S
QS1	−	S S S S
QS2	−	S S S S
QS3	−	S S S S
QS12	−	S S S S S S S
QS13	−	S S S S S S S
QS23	−	S S S S S S S
QS123	−	S S S S S S S

Fig. 1. NH_2-terminal σ^{54} mutations that lead to an enhancer-independent phenotype. Genes containing mutant σ^{54} proteins were transformed into strain YMC12, which lacks NtrC, and mutants were screened for growth on glucose-glutamine plates. The wild-type amino acid sequence 1 to 40 is shown. The mutations are changes from wild-type leucines (L) or glutamines (Q) to serines (S), as indicated in each row. Mutants that grew well are scored as (+); mutants that showed lower growth rates are scored as (+/−). The remaining mutants and wild-type σ did not grow (−), as is the case for a number of mutants that showed 90% or greater loss of activity in a NtrC-positive, σ^{54}-negative host strain (*4, 5*). The data indicate that the (+) phenotype requires at least two leucine substitutions in the boxed sequence.

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.

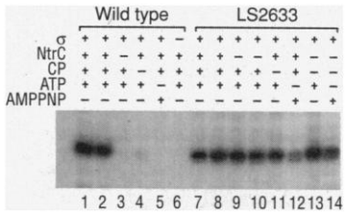

Fig. 3. Requirements for transcription by enhancer-independent mutant LS2633. Components omitted from the standard complete system are indicated and the results are compared for LS2633 and wild-type σ proteins. CP, carbamyl phosphate.

hydrolyzable form of ATP (Fig. 3) (*11*). The minimal requirements for LS2633 transcription included mutant σ^{54}, polymerase, and elongation substrates [lane 14 of Fig. 3 uses the β-γ nonhydrolyzable ATP analog adenylyl imidodiphosphate (AMPPNP)]. 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 σ^{54} 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 σ^{54} causes the polymerase to behave like σ^{70} 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 σ^{54} is the formation of a heparin-resistant (*10, 17*) open complex at the *glnAp2* promoter. Heparin-resistant *glnAp2* transcription required NtrC for wild-type σ^{54} (Fig. 5). Thus, we investigated whether LS2633 could direct heparin-resistant transcription in the absence of NtrC. The results

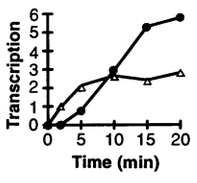

Fig. 4. Comparison of wild-type and LS2633 transcription kinetics (transcription is in arbitrary units). Wild-type reactions (●) contained NtrC and carbamyl phosphate, whereas LS2633 reactions (△) did not. All components were mixed and samples were taken at the indicated elapsed times; the amount of RNA produced at each time point was quantified with a phosphorimager and plotted.

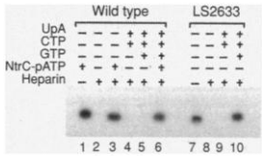

Fig. 5. Heparin-resistant transcription with LS2633. In vitro transcription reactions were preincubated, where indicated, with 0.5 mM uridylyl (3′,5′) adenosine (UpA), 0.5 mM cytidine triphosphate (CTP), 0.5 mM guanosine triphosphate (GTP), and NtrC-phosphate–ATP (NtrC-pATP) at 37°C for 5 min. Then, heparin was added (25 μg/ml final), followed immediately by the missing NTPs (0.5 mM each). After another 5 min, the reaction was stopped and the RNA was processed and visualized. The sequence of the *glnAp2* promoter from −1 to +7 is UACGGCGA.

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 σ^{54}, 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 early 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 HRS456 yielded detectable open-complex signals, which were further enhanced by the presence of NtrC. Quantitation of several experiments with the mutants indicated that ~15% of the promoter

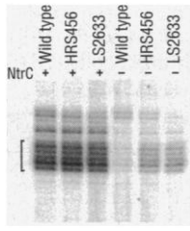

Fig. 6. Direct detection of open complexes. Transcription complexes were formed with the use of the indicated proteins (NtrC was used at 100 nM), and then the DNA was probed for single-stranded regions with permanganate. The bracket indicates the open-complex signal just upstream from the transcription start site.

DNA is in the open-complex state in the absence of NtrC, compared to less than 1% opened by the wild-type σ. Taken together with the results in Fig. 5, these data indicate that the mutations in σ^{54} 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 σ^{54} confers enhancer responsiveness to *E. coli* RNA polymerase. The NH_2-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 σ^{54} holoenzyme is the unusual ability to form an inactive stable closed complex (*2*). By contrast, σ^{70} 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 σ^{54} 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 NH_2-terminus of σ^{54} 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

1. L. J. Reitzer and B. Magasanik, *Cell* **45**, 785 (1986); J. Collado-Vides, B. Magasanik, J. D. Gralla, *Microbiol. Rev.* **55**, 371 (1991); A. K. North, K. E. Klose, K. M. Stedman, S. Kustu, *J. Bacteriol.* **175**, 4267 (1993).

2. J. D. Gralla, *Cell* **66**, 415 (1991); S. Sasse-Dwight and J. D. Gralla, *ibid.* **62**, 945 (1990); Y. Tintut, J. Wang, J. D. Gralla, *Genes Dev.* **9**, 2305 (1995).
3. C. Wong, Y. Tintut, J. D. Gralla, *J. Mol. Biol.* **236**, 81 (1994); Y. Tintut, C. Wong, Y. Jiang, M. Hsieh, J. D. Gralla, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 2120 (1994).
4. M. Hsieh and J. D. Gralla, *J. Mol. Biol.* **239**, 15 (1994).
5. M. Hsieh, Y. Tintut, J. D. Gralla, *J. Biol. Chem.* **269**, 373 (1994).
6. L. Ray, F. Claverie-Martin, P. Weglenski, B. Magasanik, *J. Bacteriol.* **172**, 818 (1990).
7. A. Syed, B. Wolner, J. D. Gralla, unpublished data.
8. Wild-type σ protein was purified as described [(D. Popham, J. Keener, S. Kustu, *J. Biol. Chem.* **266**, 19510 (1991)]. The mutant σ^{54} proteins were cloned into plasmid pJF5401 [B. Schauder, H. Blöcker, R. Grank, J. E. G. McCarthy, *Gene* **52**, 279 (1987)] and induced by shifting the temperature to 42°C for 4 hours. The mutant proteins were found in inclusion bodies, and therefore the insoluble fraction after cell disruption was taken. This fraction was dissolved in buffered 4 M guanidium HCl and 0.1% NP-40 nonionic detergent, dialyzed into 1 M guanidium and then into buffer without guanidium. Subsequent chromatography was as described for the wild-type protein. After three column purification steps, the proteins were >90% pure.
9. The standard in vitro transcription reaction contained 5 nM supercoiled DNA template pYS1 bearing the *glnAp2* promoter, 100 nM NtrC purified as described [J. B. Moore, S. P. Shiau, L. J. Reitzer, *J. Bacteriol.* **175**, 2692 (1993); the concentration used was determined by titration], 10 mM carbamyl phosphate [J. Feng *et al., ibid.* **174**, 6061 (1992)], 100 nM σ^{54}, 1 unit *E. coli* RNA core polymerase (Epicentre Technology, Madison, WI), and 0.5 mM each nucleotide triphosphate (NTP), except ATP at 3 mM, in transcription buffer [50 mM Tris (pH 7.8), 100 mM KCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, bovine serum albumin (50 ng), and 3.5% (w/v) polyethylene glycol] in a 40-μl reaction volume. All components were assembled on ice, and after addition of NTP the reactions were incubated at 37°C for 6 to 7 min. Reactions were stopped by adding an equal volume of 5 M NH_4OAc and 100 mM EDTA. The RNA transcript was ethanol-precipitated and then subjected to primer extension with reverse transcriptase (Promega) and the ^{32}P-labeled oligonucleotide downstream primer described in (*3*). The radioactive extension product was separated on a 10% urea polyacrylamide gel and visualized by autoradiography.
10. A. Ninfa, L. J. Reitzer, B. Magasanik, *Cell* **50**, 1039 (1987).
11. Twofold to threefold stimulation of transcription with σ mutants occurs at high concentrations of NtrC (J. T. Wang and J. D. Gralla, unpublished data).
12. S. Kustu, A. K. North, D. S. Weiss, *Trends Biochem. Sci.* **16**, 397 (1991); W. Wang, M. Carey, J. D. Gralla, *Science* **255**, 450 (1992).
13. D. S. Weiss, J. Batut, K. E. Klose, J. Keener, S. Kustu, *Cell* **67**, 155 (1991).
14. Standard in vitro transcription reactions were assembled except for LS2633, where NtrC, carbamyl phosphate, and the high concentration of ATP were omitted. Samples were taken from the reaction at various times. The primer extension product of the RNA was visualized and quantified by direct phosphorimaging.
15. W. Su, S. Porter, S. Kustu, H. Echols, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 5504 (1990).
16. C. A. Gross, M. Lonetto, R. Losick, in *Transcriptional Regulation,* S. L. McKnight and K. R. Yamamoto, Eds. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1992), pp. 129–176.
17. D. L. Popham, D. Szeto, J. Keener, S. Kustu, *Science* **243**, 629 (1989).
18. Y. Tintut, J. T. Wang, J. D. Gralla, *J. Biol. Chem.* **270**, 24392 (1995).
19. Potassium permanganate footprinting was done under the conditions of in vitro transcription, except that the supercoiled DNA template was at 1 nM, KCl was at 20 mM, and Hepes replaced Tris buffer. Other details are as described in (*18*).
20. We thank A. Ninfa and J. Moore for expression plasmids; Y. Tintut, H. Cai, and Y. Song for their help; and A. J. Courey and J. Gober for advice. Supported by NIH grant GM35754. M.H. was supported in part by grants NSC84-2331-B-040-008 and CSMC 83-NS-A-047 from the government of Taiwan.

7 June 1995; accepted 14 September 1995

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 dependence 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 remodeled to allow gene 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 dihydrofolate reductase (DHFR) gene, even though some sites may be preferred (*2–4*). Several other studies have suggested a lack of specific origin sequences 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 δ- and β-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*).

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'Ulm, 75230 Paris Cedex 05, France.

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-kbp intervals, in the rDNA of *Xenopus* early blastulae (*12*). Plasmids containing the *Xenopus* rDNA sequences also showed random initiation and termination of DNA replication in *Xenopus* eggs or egg extracts (*13*, *14*). An important difference with the other experimental systems is that transcription of the zygotic genome, including rRNA genes (*15*), is repressed in eggs and early embryos. In order to analyze the relation between transcription and replication at this locus, we investigated whether resumption of rRNA synthesis after the *Xenopus* mid-blastula transition is accompanied by changes in chromosomal rDNA replication.

RIs of the restriction fragments (Fig. 1A) were analyzed by 2D gel electrophoresis (Fig. 1C). The principle of this technique is to resolve replication fork–containing restriction fragments according to both mass and shape (*16*) (Fig. 1B). The relative amounts of bubbles, double forks, and simple forks of a given fragment reflect the frequencies of initiation and termination within that fragment.

At the mid-blastula stage, each restriction fragment showed a strong bubble arc, a simple fork arc, and a double fork triangular smear, indicating that initiation and termination occur at multiple positions throughout rDNA repeats. These patterns are essentially similar to those we found previously for the early blastula stage (*12*).

At later stages, the ratio of bubbles to simple forks was visibly decreased in fragments B, C, D, and E but not in fragment A. RIs were measured on a PhosphorImager