[53] Enhancer-Dependent Transcription by Bacterial RNA Polymerase: The β Subunit Downstream Lobe Is Used by σ^{54} During Open Promoter Complex Formation

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The DNA-dependent RNA polymerase (RNAP; EC 2.7.7.6) of *Escherichia coli*, the best-characterized multisubunit RNAP, is composed of a core enzyme (E, subunit composition $\alpha_2\beta\beta'\omega$) and one of seven identified molecular species of the σ subunit (Eσ, subunit composition $\alpha_2\beta\beta'\omega\sigma$). Advances in high-resolution structural analysis of the bacterial RNAP have opened up opportunities to study the functional role that each structural module of the RNAP plays in transcription.[1,2] A mobile structural module of *E. coli* RNAP, known as the β downstream lobe (residues 186–433), was shown to contribute to stable open promoter complex formation during transcription directed by RNAP containing the σ^{70} factor.[3] This article describes experimental systems used to probe the function of the β subunit downstream lobe in the context of RNAP containing the major variant σ subunit, the enhancer-dependent σ factor, σ^{54}.[4] Both enhancer-dependent RNAP (σ^{54}–RNAP) and enhancer-independent RNAP (σ^{70}–RNAP) are capable of promoter recognition that results in the formation of the closed promoter complex. σ^{70}–RNAP closed promoter complexes can isomerize rapidly into transcriptionally active open promoter complexes in the absence of additional activators or energy sources. In contrast, σ^{54}–RNAP complexes remain closed unless an enhancer DNA-bound activator and an energy source in the form of ATP or GTP hydrolysis is provided. The ATPase activity of the activator induces the propagation of initial DNA melting or distortion in the closed σ^{54}–RNAP promoter complexes toward the transcription initiation start point and allows open promoter complex formation.[5,6]

[1] K. S. Murakami, S. Masuda, and S. A. Darst, *Science* **296,** 1280 (2002).
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[3] S. Nechaev, M. Chlenov, and K. Severinov, *J. Biol. Chem.* **275,** 25516 (2000).
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Preparation of Proteins

Overexpression and Purification of Recombinant σ^{54}

For historical reasons, we used the σ^{54} protein from *Klebsiella pneumoniae*. The σ^{54} from *K. pneumoniae* and *E. coli* are essentially interchangeable and have no significantly different properties. *K. pneumoniae* σ^{54} can be expressed to high levels in *E. coli* cells using the T7 RNAP–T7 promoter system. Plasmid pSRWσ^{54}, which contains the *K. pneumoniae* σ^{54} under the control of the T7 promoter, is transformed into *E. coli* strain B834(DE3) (Novagen). Freshly transformed cells are used to inoculate 1 liter of LB medium (50 cfu/liter) containing kanamycin (50 μg/ml) and are grown at 37° with vigorous shaking in a 2-liter flask. At $OD_{600} = 0.5$, the culture is shifted to a 25° water bath and shaken for an additional 30 min before induction by the addition of isopropylthio-β-D-galactosidase (IPTG) to 1 m*M*. After 3-h induction at 25°, cells are harvested by centrifugation (10,000 rpm; 10 min at 4° in a Beckman JA-14 rotor), resulting in 1–2 g of a wet cell pellet, which can be stored at −80°. For lysis, the cell pellet is resuspended in 20 ml of ice-cold lysis buffer [10 m*M* Tris–HCl, pH 8.0, 50 m*M* NaCl, 1 m*M* dithiothreitol (DTT), 0.1 m*M* ethylenediaminetetraacetic acid (EDTA), and 5% (v/v) glycerol] containing a cocktail tablet of protease inhibitors (Complete, Roche Diagnostics) and lysed in a cell disrupter (Constant Systems Ltd., UK). The cell lysate is centrifuged at 18,000 rpm in a Beckman JA-20 rotor at 4° for 30 min to remove cell debris and inclusion bodies. The overexpressed σ^{54} is usually in the soluble cell fraction, and the supernatant can be used directly for the purification steps. The supernatant is transferred to a fresh centrifuge tube, and streptomycin sulfate (Sigma) [from a 20% (v/v) stock] is added gradually to a final concentration of 2.0% (v/v) at 4° and incubated while stirring gently on ice for 30 min. The streptomycin sulfate precipitate is then recovered by centrifuging at 18,000 rpm in a Beckman JA-20 rotor for 30 min at 4°. The resulting supernatant is transferred to a fresh centrifuge bottle, and solid ammonium sulfate is added slowly to final concentration of 70% (w/v) and incubated while stirring gently at 4° on ice for 30 min. The ammonium sulfate precipitate is centrifuged (18,000 rpm; 30 min at 4° in a Beckman JA-20 rotor). The resulting pellet is then resuspended in 5 ml of Sepharose buffer A [20 m*M* imidazole, pH 7.0, 100 m*M* NaCl, and 5% (v/v) glycerol] and dialyzed overnight at 4° in Sepharose buffer A. The dialysate is centrifuged (18,000 rpm; 30 min at 4° in a Beckman JA-20 rotor) and loaded onto a preequilibrated (according to manufacturers' instructions) 5 ml Hi-Trap Q Sepharose column (Amersham Biosciences) using a fast protein liquid chromatography (FPLC) machine (Amersham Biosciences). The

column is washed with 100 ml of Sepharose buffer A. The σ^{54} is eluted (in 1-ml fractions) with a 200-ml linear gradient of 0 to 1 *M* NaCl in Sepharose buffer A. Fractions are analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and fractions containing the σ^{54} are pooled and dialyzed overnight against heparin buffer A [10 m*M* Tris–HCl, pH 8.0, 50 m*M* NaCl, 1 m*M* DTT, 0.1 m*M* EDTA, and 5% (v/v) glycerol] at 4°. The dialysate is centrifuged (18,000 rpm; 30 min at 4° in a Beckman JA-20 rotor). The resulting supernatant is transferred to a fresh centrifuge tube, and solid $MgCl_2$ is added to a final concentration of 10 m*M* and stirred gently until all the $MgCl_2$ crystals are dissolved. The supernatant is then loaded onto a preequilibrated (according to manufacturers' instructions) 5-ml Hi-Trap heparin column (Amersham Biosciences) using an FPLC (Amersham Biosciences) to separate σ^{54} from the core RNAP. The column is washed with 100 ml of heparin buffer A. The σ^{54} is eluted (in 1-ml fractions) with a 200-ml gradient of 0 to 1 *M* NaCl in heparin buffer A containing 10 m*M* $MgCl_2$. Fractions are analyzed by SDS–PAGE and those containing pure σ^{54} are pooled and dialyzed overnight against storage buffer [10 m*M* Tris–HCl, pH 8.0, 50 m*M* NaCl, 0.1 m*M* EDTA, 1 m*M* DTT and 50% (v/v) glycerol] at 4°. Aliquots of σ^{54} are stored either at −20° (short-term) or at −80° (long term). The typical yield is about 5–10 mg (protein estimated by Bradford assay[7] using bovine serum albumin as standard).

Overexpression, Purification, and In Vivo *Reconstitution of Hexahistidine-Tagged Wild-Type and* β*(Δ186–433) Core RNAP*

Plasmids expressing the hexahistidine-tagged (6His-tagged) β subunit, either wild-type or lacking residues 186–433, a nonessential, evolutionarily variable domain of *E. coli* RNAP that forms the RNAP downstream lobe and is required for the action of termination factor Alc,[8] are transformed into *E. coli* strain XL-1 blue (Stratagene). Freshly transformed cells are used to set up a 200-ml starter culture that is grown overnight at 37° in LB medium containing ampicillin (200 μg/ml) in a 1-liter flask. For overexpression, four 1-liter batches of LB medium are inoculated with 50 ml of starter culture and grown at 37° with ampicillin (200 μg/ml) with vigorous shaking in 2.5-liter flasks. At $OD_{600} = 0.4$, expression of the β subunit gene is induced by the addition of IPTG at a final concentration of 1 m*M* and growth is continued for another 3–4 h. Cells are harvested by centrifugation (5000 rpm; 10 min at 4° in a Sorvall GS-3 rotor). If not used

[7] M. Bradford, *Anal Biochem*. **72,** 248 (1976).
[8] K. Severinov, M. Kashlev, E. Severinova, I. Bass, K. McWilliams, E. Kutter, V. Nikiforov, L. Snyder, and A. Goldfarb, *J. Biol. Chem*. **269,** 14254 (1994).

immediately, the cell pellet can be stored at −80°. The cell pellet is resuspended in 30 ml of grinding buffer [50 m*M* Tris–HCl, pH 8.0, 200 m*M* NaCl, 5 m*M* β-mercaptoethanol, 1 m*M* EDTA, 1 m*M* phenylmethylsulfonyl fluoride (PMSF) and 5% (v/v) glycerol] and disrupted by sonication, and the lysate cleared by centrifugation (15,000 rpm; 15 min at 4° in a Sorvall SS-34 rotor). The supernatant is transferred to a centrifuge tube, polymin P (Kodak) [from a 10% (v/v) stock made in H_2O, pH adjusted to 8.0 with HCl] is added gradually to a final concentration of 0.8% (v/v) at 4°, and the mixture is incubated on ice for 10 min. The precipitate, which is formed upon polymin P addition, is collected by centrifugation (5000 rpm; 5 min at 4° in a Sorvall SS-34 rotor), and the pellet is washed in 20 ml of buffer A [10 m*M* Tris–HCl, pH 8.0, 500 m*M* NaCl, 5 m*M* β-mercaptoethanol, and 5% (v/v) glycerol] by brief sonication followed by centrifugation (10,000 rpm; 15 min at 4° in a Sorvall SS-34 rotor). The washed pellet is resuspended carefully in ~0.5 ml of buffer A containing 1 *M* NaCl using a glass rod, followed by further addition of buffer A containing 1 *M* NaCl to a 20-ml final volume and brief sonication to ensure that a homogeneous suspension is obtained. Following centrifugation (10,000 rpm; 5 min at 4° in a Sorvall SS-34 rotor), the pellet is resuspended carefully as before. Supernatants from the two 1 *M* NaCl wash steps (~40 ml total volume) are transferred into a fresh centrifuge bottle, and proteins are precipitated by the addition of ammonium sulfate powder to 70% saturation (0.45 g/ml of supernatant). Proteins, which include RNAP, are allowed to precipitate for 1 h or, preferably, overnight at 4°. Precipitated proteins are collected by centrifugation (15,000 rpm; 30 min at 4° in a Beckman JA-20 rotor) and resuspended in 30 ml of buffer A (containing no NaCl). After centrifugation (15,000 rpm; 30 min at 4° in a Sorvall SS-34 rotor), the supernatant is loaded onto a 5-ml Heparin Hi-Trap column (Amersham Biosciences) equilibrated with buffer A (containing 100 m*M* NaCl) and connected to an FPLC system. After loading, the column is washed with 15 ml of the same buffer. Bound proteins are eluted with three steps of increased concentrations of NaCl in buffer A (0.3, 0.6, and 1.0 *M*). Eluting proteins are monitored by UV absorbance (OD_{260}), and entire peaks are collected in a single vessel. Fractions are analyzed by SDS–PAGE, and the RNAP-containing fraction (usually, the 0.6 *M* NaCl fraction) is supplemented with 2 m*M* imidazole and loaded onto a precharged (with $NiCl_2$) and preequilibrated (according to manufacturers' instructions) 1-ml Hi-Trap chelating HP column (Amersham Biosciences) connected to an FPLC system (Amersham Biosciences). Nonspecifically bound proteins are removed by washing the column with 3 ml of buffer A containing 500 m*M* NaCl and 2 m*M* imidazole, followed by washing the column with 10 ml buffer A containing 500 m*M* NaCl and 20 m*M* imidazole. RNAP is eluted with buffer A

containing 500 m*M* NaCl and 200 m*M* imidazole. RNAP-containing fractions are identified by SDS–PAGE. The Ni chromatography step allows the separation of RNAP containing the plasmid-borne β subunit from chromosomally encoded, wild-type RNAP and is critical for preparation of mutant RNAP free of contaminating wild-type enzyme. Because the mutant β subunit is substantially smaller than the wild-type β subunit, a 6–8% (w/v) SDS–PAGE is sufficient to screen for fractions eluted by 200 m*M* imidazole buffer containing equimolar amounts of RNAP β' and shortened β subunit, which do not contain the full-length β subunit. In contrast, the wash fractions should contain equimolar amounts of full-sized β and β' and no shortened β. The 200 m*M* imidazole fraction contains highly pure RNAP, which is a mixture of core RNAP and σ^{70} RNAP holoenzyme. To obtain pure core RNAP enzyme, the 200 m*M* imidazole fraction is diluted threefold in buffer A and loaded onto a 1-ml ProteinPak HQ column (Waters) preequilibrated (with buffer A containing 200 m*M* NaCl) and attached to an FPLC. After loading, the column is washed with 2–3 ml of buffer A containing 200 m*M* NaCl, and RNAP is eluted with a 10-ml linear gradient (from 200 to 400 m*M* NaCl) in buffer A. RNAP core and RNAP holoenzyme are eluted at about 320 and 340 m*M* NaCl, respectively. Fractions are analyzed by SDS–PAGE, and peak fractions containing pure core RNAP are pooled and concentrated by ultrafiltration through a Centricon-100 centrifugal concentrator (Amicon) to approximately 1 mg/ml (estimated by Bradford assay[7] using bovine serum albumin as standard). Glycerol is added to 50% (v/v), and core RNAP is aliquoted and stored at -20°. The typical yield is approximately 200 μg of mutant core RNAP from a 4-liter cell culture.

Overexpression and Purification of 6His-Tagged E. coli σ^{54} *Activator Phage Shock Response Protein F (PspF)*

To assess the activities of wild-type and mutant Eσ^{54}, we use two forms of the *E. coli* σ^{54} activator PspF, namely PspF and $PspF_{1-275}$. $PspF_{1-275}$ lacks the carboxyl-terminal DNA-binding domain and essentially represents the central catalytic domain of σ^{54} activators and has the advantage of being able to activate the Eσ^{54} without the requirement for an enhancer sequence, hence simplifying the design of activity assays. *pspF* and *pspF1–275* genes are cloned into pET28b^{+} (Novagen) and expressed using the T7 RNAP–T7 promoter system. The encoded proteins (PspF and $PspF_{1-275}$, respectively) contain an amino-terminal 6His tag and a site for thrombin-mediated cleavage for the removal of the 6His tag if desired. The culturing procedure used to overexpress $PspF_{1-275}$ is identical to that used for σ^{54} (see earlier discussion). The cell pellet is resuspended in 25 ml of ice-cold

Ni buffer A [25 m*M* NaH_2PO_4, pH 7.0, 500 m*M* NaCl, and 5% (v/v) glycerol] containing a cocktail tablet of protease inhibitors (Complete, Roche Diagnostics) and lysed in a cell disrupter (Constant Systems Ltd., UK). The cell lysate is centrifuged at 18,000 rpm in a Beckman JA-20 rotor at 4° for 30 min. The overexpressed 6His-tagged $PspF_{1–275}$ is usually in the soluble cell fraction, and the supernatant can be used directly for the affinity purification step. For affinity purification, 25 ml of the supernatant is loaded onto a precharged (with $NiCl_2$), preequilibrated (according to manufacturers' instructions) 5-ml Hi-Trap chelating HP column (Amersham Biosciences) using an FPLC machine (Amersham Biosciences). Nonspecifically bound proteins are removed by washing the column with 50 ml of 0.05 *M* imidazole in Ni buffer A. The 6His-tagged $PspF_{1–275}$ is eluted (in 1-ml fractions) with a 40-ml linear gradient of 0.05 to 0.8 *M* imidazole in Ni buffer A. Fractions are analyzed by SDS–PAGE, and fractions containing the 6His-tagged $PspF_{1–275}$ are pooled and dialyzed overnight against storage buffer [10 m*M* Tris–HCl, pH 8.0, 50 m*M* NaCl, 0.1 m*M* EDTA, 1 m*M* DTT, and 50% (v/v) glycerol] at 4°. Aliquots of 6His-tagged $PspF_{1–275}$ are stored at −80°. The typical yield is about 5–10 mg (protein estimated by Bradford assay[7] using bovine serum albumin as standard), and typical purity is >98%.

Full-length PspF can be overproduced using *E. coli* strain B834(DE3), but in less than optimal quantities. Further, using *E. coli* strain B834(DE3) as the expression host limits the solubility of the overexpressed 6His-tagged PspF. In order to maximize the amount of soluble over expressed 6His-tagged PspF, a different expression host was used, *E. coli* strain C41,[9] which has uncharacterized mutations that allow higher-level expression of proteins that are toxic. For overexpression, a freshly transformed colony of *E. coli* strain C41 harboring plasmid pET28b$^+$–*pspF* is used to inoculate 10 ml of LB medium containing kanamycin (25 μg/ml) (starter culture). Following overnight growth at 37°, two 1-liter aliquots of LB medium in 2.5-L flasks are inoculated with 1 ml of the starter culture and grown at 37° with vigorous shaking. At $OD_{600} = 0.4$, the culture is shifted to a 16° water bath and shaken for an additional 30 min before induction by the addition of IPTG to 0.5 m*M*. After 12–16 h at 16°, the culture is harvested by centrifugation (10,000 rpm; 10 min at 4° in a Beckman JA-14 rotor), resulting in ~10 g of a wet cell pellet, which can be stored at −80°. For lysis, the cell pellet is resuspended in 10 ml of ice-cold Ni buffer A containing a cocktail tablet of protease inhibitors (Complete, Roche Diagnostics), processed, and purified as described for $PspF_{1–275}$ by FPLC chromatography using a Hi-Trap chelating HP column (Amersham Biosciences).

[9] B. Miroux and J. E. Walker, *J. Mol. Biol.* **260,** 289 (1996).

Peak fractions are pooled and dialyzed overnight against PspF storage buffer [10 m*M* Tris–HCl pH 8.0, 500 m*M* NaCl, 0.1 m*M* EDTA, 1 m*M* DTT, and 50% (v/v) glycerol] at 4°. Aliquots of PspF are stored at −80°. The typical yield is about 5–6 mg (protein estimated by Bradford assay[7] using bovine serum albumin as standard), and typical purity is >95%.

Characterization of σ^{54}-RNAP

In Vitro *Reconstitution of* σ^{54}*-RNAP*

While many methods exist for assessing RNAP holoenzyme formation *in vitro*, the native gel assembly assay, a semiquantitative assay, has often been the method of choice for convenient and quick analysis of σ^{54}–RNAP formation and the effect of the $\beta(\Delta 186–433)$ mutation on holoenzyme formation. In this assay, σ^{54}, core RNAP, and holoenzyme are resolved on a native gel, and holoenzyme formation is judged by depletion of the bands corresponding to the core RNAP and the appearance of a new, faster, migrating band corresponding to the holoenzyme. Binding reactions are conducted in core binding buffer [40 m*M* Tris–HCl, pH 8.0, 100 m*M* NaCl, 1 m*M* DTT, 0.1 m*M* EDTA, and 10% (v/v) glycerol]. First, core RNAP (250 n*M*) and different amounts of σ^{54} (at typical molar ratios of 1:1 to 1:4 of core RNAP to σ^{54}) are mixed together at 4° in a final reaction volume of 10 μl. Following incubation at 30° for 10 min, 2 μl of native-loading dye [from a 5 × stock: core binding buffer containing 50% (v/v) glycerol and 0.05% (w/v) bromphenol blue] is added to each reaction. For electrophoresis, samples are loaded onto 4.5% (w/v) native polyacrylamide Bio-Rad Mini-Protean gels. The gels are run in Tris–glycine buffer (25 m*M* Tris and 200 m*M* glycine, pH 8.6) at 50 V for 2 h at room temperature. The electrophoresed proteins are visualized by staining and destaining the gels with Coomassie blue dye [0.5% (w/v) Coomassie blue (Sigma), 50% (v/v) methanol, and 7.5% (v/v) acetic acid] and destain solution [45% (v/v) methanol and 9% (v/v) acetic acid], respectively. A representative native gel is shown in Fig. 1 in which the binding activity of wild-type and $\beta(\Delta 186–433)$ core RNAP to σ^{54} is compared.

Promoter Complex Formation by σ^{54}*-RNAP*

To monitor the contribution of RNAP surfaces to σ^{54}-dependent promoter complex formation, an electrophoretic mobility shift assay (EMSA) is used. The promoter DNA probe is prepared using two fully complementary (for homoduplex probe; Fig. 2) 88-bp oligonucleotides that comprise the *Sinorhizobium meliloti nifH* promoter sequence (from positions −60

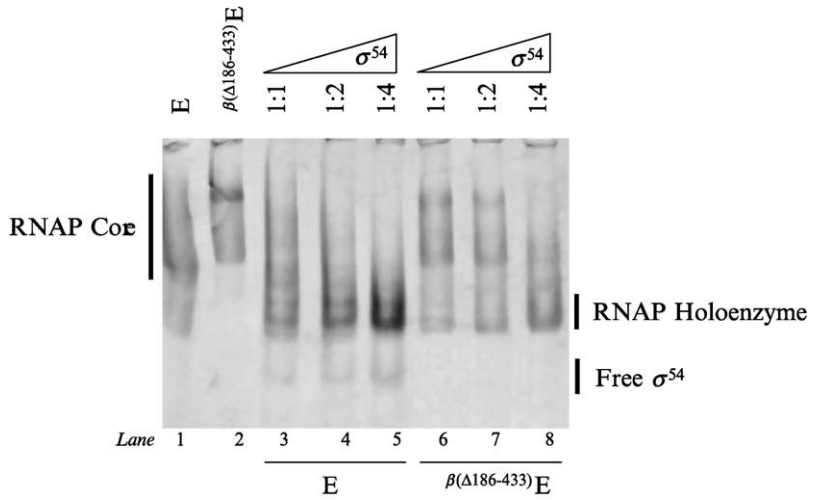

FIG. 1. Natively gel [4.5% (w/v)] showing the binding of increasing amounts of σ^{54} (250–1000 n*M*) to free wild-type (E) and $\beta(\Delta 186–433)$ core RNAP (lanes 1 and 2, respectively) to form the RNAP holoenzyme (lanes 3–5 and 6–8, respectively). The migration positions of core RNAP, free σ^{54}, and holoenzyme are indicated.

to +28 with respect to the transcription start site at +1). Heteroduplex variants of promoters are used routinely to study the interactions made by the RNAP during open complex formation and promoter escape.[10] Two heteroduplex promoter probes often used to assess and dissect the contribution of σ^{54} and RNAP to promoter complex formation by the σ^{54}–RNAP *en route* to transcription initiation are the early melted and late melted promoter probes (Fig. 2). Early melted and late melted promoter probes represent the conformation of promoter DNA as it is thought to exist within the closed and open σ^{54}–RNAP promoter complexes, respectively. Like the homoduplex promoter probe, heteroduplex promoter probes are made using two 88-bp oligonucleotides comprising the *S. meliloti nifH* promoter, with one of the oligonucleotides (nontemplate strand) containing the region of mismatched sequences to generate the heteroduplex segment (Fig. 2). Thus, in the case of early melted and late melted promoter probes, positions -12 and -11 and positions −10 to −1 are mismatched, respectively.

Preparation of Homoduplex and Heteroduplex Promoter Probes. Oligonucleotides are purchased from MWG-Biotech AG, Germany. For heteroduplex formation, sequences are chosen in order to prevent

[10] W. Cannon, S. R. Wigneshweraraj, and M. Buck, *Nucleic Acids Res.* **30,** 886 (2002).

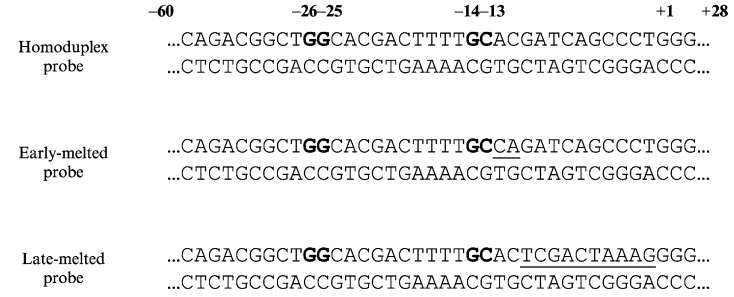

FIG. 2. *Sinorhizobium meliloti nifH* homoduplex and heteroduplex (early melted and late melted) promoter probes used for EMSAs. The consensus GG and GC elements of enhancer-dependent promoters are in bold, and their positions with respect to the transcription start site at +1 are given. Mutant sequences introduced in the nontemplate strand to create the heteroduplex segments are underlined.

purine–pyrimidine mismatched base pairing. Prior to duplex formation, one of the oligonucleotides is ^{32}P end labeled at the 5′ end. End labeling is conducted in 20-μl reactions in T4 polynucleotide kinase buffer (One-Phor-All buffer, Amersham Biosciences) containing 20 pmol of oligonucleotide, 35 μCi of [γ-^{32}P]ATP (10 mCi/ml), and 10 U of T4 polynucleotide kinase (Amersham Biosciences). Following incubation at 37° for 30 min, the reaction is incubated further at 70° for 10 min to inactivate the T4 polynucleotide kinase. For duplex formation, respective pairs of oligonucleotides with the unlabeled strand present at a twofold molar excess (10 pmol in 20 μl) are incubated at 95° for 3 min in TM buffer (10 m*M* Tris–HCl, pH 8.0, and 10 m*M* $MgCl_2$) and then chilled rapidly in iced water for 5 min to allow duplex formation.

Assays to Measure Closed and Open Promoter Complex Formation by σ^{54}–RNAP. Reactions are conducted in STA buffer [25 m*M* Tris–acetate, pH 8.0, 8 m*M* Mg-acetate, 10 m*M* KCl, 1 m*M* DTT, and 3.5% (w/v) polyethylene glycol (PEG-6000, Sigma)]. Simple binding assays for σ^{54}–RNAP closed promoter complex formation contained a 16 n*M* ^{32}P-labeled homo- or heteroduplex probe and 100 n*M* σ^{54}–RNAP (assembled using a 1:4 molar ratio of core RNAP to σ^{54}) in a final volume of 10 μl. Reactions are incubated at 37° for 5 min and stopped by adding 2 μl of native-loading dye. For electrophoresis, 4 μl of the reaction is loaded onto 4.5% (w/v) native polyacrylamide Bio-Rad Mini-Protean gels. The gels are run in TBE (89 m*M* Tris, 89 m*M* *ortho*-boric acid, and 2 m*M* EDTA) buffer at 60 V for 80 min at room temperature. To assess open promoter complex formation, reactions are composed essentially as described earlier using the homoduplex promoter probe. Following incubation at 37° for 5 min,

4 μM $PspF_{1–275}$ and 1 mM ATP or GTP are added to the reaction and incubated further for 5 min at 37° to activate open or initiated promoter complex formation, respectively. (*Note*. GTP allows the synthesis of a 3-bp transcript from the *S. meliloti nifH* promoter; Fig. 2.) Closed σ^{54}–RNAP promoter complexes are sensitive to heparin, whereas open or initiated σ^{54}–RNAP promoter complexes are resistant. Therefore, to destroy closed and unstable promoter complexes, the reactions are stopped by adding native-loading dye containing heparin to a final concentration of 100 μg/ml and, following incubation at 37° for 5 min, electrophoresed as described previously. The gels are dried and quantified using a PhosphorImager (BAS-1500, Fuji, Japan).

Assays with Heteroduplex Promoter Probes. Wild-type σ^{54}–RNAP promoter complexes formed on the early melted promoter probe are resistant to heparin, in contrast to those formed on homoduplex promoter probes in the absence of activation or initiation.[10] This stability is mainly attributed by the ability of the σ^{54}–RNAP to recognize and bind tightly to the DNA fork junction structure present within the early melted promoter probe; this interaction is of regulatory significance for transcription activation by σ^{54} activators.[6,10] Wild-type σ^{54}–RNAP promoter complexes formed on the late melted promoter probe are sensitive to heparin and only become heparin resistant following activation (with $PspF_{1–275}$ and ATP) or initiation (using $PspF_{1–275}$ and GTP).[11] This suggests that conformational changes must occur within the σ^{54}–RNAP for it to acquire heparin stability on the late melted promoter probe.[11] Therefore, EMSAs are designed and conducted (exactly as described earlier) using these heteroduplex promoter probes to assess the contribution the β downstream lobe makes toward (i) early DNA interactions made by the σ^{54}–RNAP and (ii) the acquisition of heparin stability in response to activation.

In Vitro *Transcription Assays*

One of the most common *in vitro* assays used to assess open complex formation is the single-round transcription assay. In this assay a plasmid harbors the promoter, to which the RNAP binds, initiates transcription, and transcribes until it encounters a factor-independent transcription termination sequence. Transcription elongation is conducted in the presence of heparin, which prevents reinitiation and thus results in a single round of transcription from the promoter. In the case of σ^{54}-dependent transcription, the single-round transcription assay is used mainly to check whether mutant forms of σ^{54}–RNAP are able to form activator-dependent open

[11] W. Cannon, M. T. Gallegos, P. Casaz, and M. Buck, *Genes Dev*. **13,** 357 (1999).

promoter complexes (regulated transcription assay) and whether the mutations have resulted in an activator-independent σ^{54}–RNAP that transcribes via an unstable open complex (deregulated transcription assay).

Regulated Transcription Assay. Standard reaction mixtures contain 100 n*M* σ^{54}–RNAP (reconstituted with 1:4 ratio RNAP to σ^{54}) and 20 U of RNase inhibitor (RNasin from Promega, UK) in STA buffer in a final reaction volume of 10 μl. Reactions are incubated at 37° for 5 min, and template DNA is added to a final concentration of 20 n*M*. For activation from solution using $PspF_{1–275}$, plasmid pMKC28,[12] which harbors the *S. meliloti nifH* promoter, is used as the template. For activation from an enhancer-bound activator using full-length PspF, plasmid pSLE1,[13] which contains the σ^{54}-dependent *pspA* promoter and a PspF-specific upstream enhancer region, is used as the template. [*Note*. Plasmid pTE103,[14] the parent vector for pMKC28 and pSLE1, contains the phage T7 early transcriptional terminator sequence downstream of the multiple cloning site region (MCS). Promoters inserted into the MCS of pTE103 direct transcription clockwise (5′ to 3′ direction) to generate a discrete transcript of ~470 bp.] Following incubation at 37° for 5 min, 2 m*M* ATP and 4 μ*M* $PspF_{1–275}$ or 100 n*M* PspF are added to initiate open complex formation for 5 min at 37°. For transcript production, 2 μl of a master mix containing final concentrations of 1 m*M* ATP, CTP, and GTP, 0.5 m*M* UTP, 3 μCi of [α-^{32}P]UTP (20 mCi/ml), and 100 μg/ml heparin are added and incubated further for 5 min at 37°. The reaction is stopped by adding 4 μl of formamide-loading dye [3% (w/v) xylene, 3% (w/v) bromophenol blue, and 800 μl of 250 m*M* EDTA in 10 ml of deionized formamide]. The reaction is heated at 90° for 1 min and put on ice. Half (7 μl) of the reaction is used for electrophoresis on a 6% (w/v) polyacrylamide–6 *M* urea gel. Following electrophoresis, the discrete ^{32}P-labeled RNA product can be visualized and quantified by PhosphorImager (BAS-1500, Fuji, Japan) analysis of the dried gel. A representation of a 6% (w/v) denaturing gel used for analyzing activator-dependent transcription by the wild-type and β(Δ186–433) RNAP is shown in Fig. 3.

Deregulated Transcription Assay. For activator-independent transcription by σ^{54}–RNAP, the reactions are composed as described earlier for the regulated transcription assay, but pMKC28 is used as the preferred template, the activator is omitted, and a form of σ^{54} altered in its regulatory properties is used.[4] Following incubations to reconstitute the σ^{54}–RNAP

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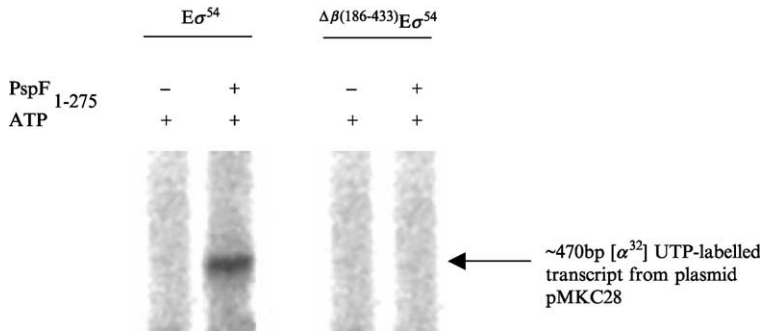

FIG. 3. Section of a 6% (w/v) denaturing gel showing that β subunit residues 186–433 are required for regulated (activator-dependent) transcript formation from the plasmid pMKC28 containg the *S. meliloti nifH* promoter.

and allow closed complexes to form, 4 m*M* GTP is added to allow activator-independent initiation to occur from the *S. meliloti nifH* promoter (Fig. 2). The reaction is then processed and the transcript is analyzed exactly as described for the regulated transcription assay.

Concluding Remarks

The protocols described in this article provide a simple step-by-step guide to obtain a relatively pure preparation of proteins required to study enhancer-dependent transcription. The experimental assays are designed to measure *in vitro* reconstitution of the σ^{54}–RNAP and to assess the extent of DNA opening (open complex formation) by the σ^{54}–RNAP in response to activation. We have used these assays in conjunction with potassium permanganate probing[15] of wild-type and $\beta(\Delta 186–433)E\sigma^{54}$–RNAP promoter complexes to show that RNAP β subunit residues 186–433 are used commonly by the enhancer-independent $E\sigma^{70}$ and enhancer-dependent $E\sigma^{54}$ for open promoter complex formation *en route* to transcription initiation.[3,16]

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