

# RNA Polymerase $\beta'$ Subunit: A Target of DNA Binding-Independent Activation

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The bacteriophage N4 single-stranded DNA binding protein (N4SSB) activates transcription by the *Escherichia coli*  $\sigma^{70}$ -RNA polymerase at N4 late promoters. Here it is shown that the single-stranded DNA binding activity of N4SSB is not required for transcriptional activation. N4SSB interacts with the carboxyl terminus of the RNA polymerase  $\beta'$  subunit in a region that is highly conserved in the largest subunits of prokaryotic and eukaryotic RNA polymerases.

N4SSB, a single-stranded DNA (ssDNA) binding protein encoded by bacteriophage N4, activates transcription by the *Escherichia coli*  $\sigma^{70}$  RNA polymerase (RNAP) at N4 late promoters (1). In addition, N4SSB is required for N4 replication (2) and recombination (3). N4SSB does not detectably bind double-stranded DNA (dsDNA) (2). Therefore, the mechanism of N4SSB transcriptional activation involves its ssDNA binding activity (for example, to facilitate isomerization from a closed complex to an open one), or protein-protein interactions with RNAP, or both.

In the course of a systematic mutational analysis of N4SSB (4), we found that its ssDNA binding and transcriptional activation functions are separable. We identified two N4SSB derivatives, Y75A and Y128A (5), that are fully functional in transcriptional activation (Fig. 1A) but defective in ssDNA binding, N4 recombination, and N4 replication (Fig. 1, B to D) (6, 7). We also identified two N4SSB derivatives,  $\Delta 264$ -265;S260A and K264A;K265A (5), with the reciprocal phenotype, that is, defective in transcriptional activation (Fig. 1A) but fully functional in ssDNA binding, recombination, and replication (Fig. 1, B to D). On the basis of these data, we infer that the ssDNA binding activity of N4SSB is not required for transcriptional activation. We infer further that the residues Ser<sup>260</sup>, Lys<sup>264</sup>, and Lys<sup>265</sup> in the COOH-terminus of N4SSB constitute part or all of an "activating region" required for transcriptional activation, and we propose that this activating region makes direct protein-protein in-

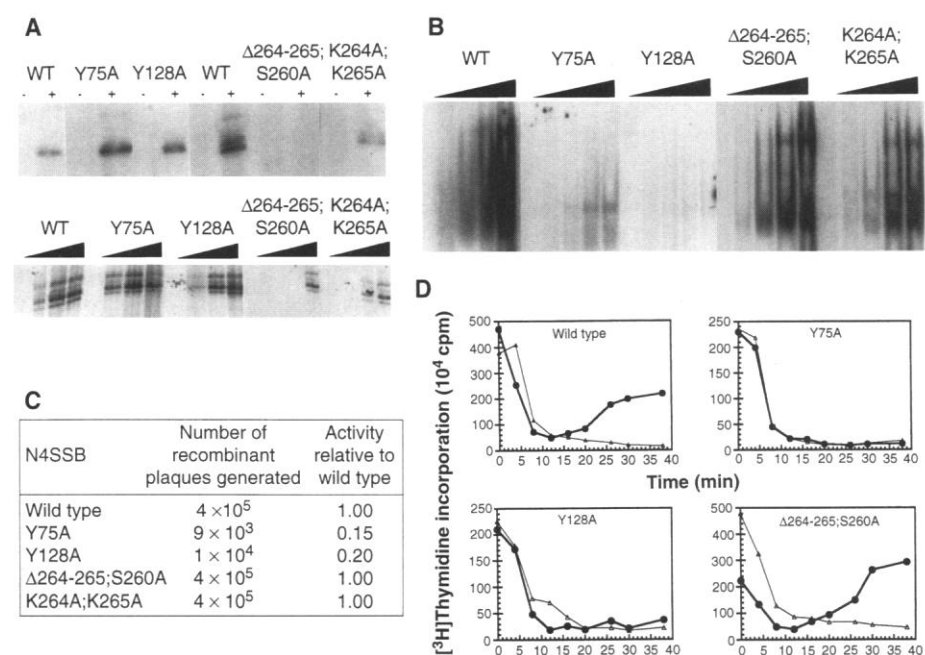
teractions with RNAP.

To test our hypothesis that N4SSB activates transcription by interacting with RNAP, we assayed interactions between N4SSB and immobilized RNAP holoenzyme ( $\alpha_2\beta\beta'\sigma^{70}$ ), RNAP core ( $\alpha_2\beta\beta'$ ), and the RNAP subassembly  $\alpha_2\beta$  (7-9) (Fig. 2A). Wild-type N4SSB was retained by immobilized holoenzyme and core, even in the presence of 1.5 M NaCl, but it was not retained by immobilized  $\alpha_2\beta$  (Fig. 2A). The interaction was specific. The interactions of N4SSB derivatives functional in transcriptional activation but defective in ssDNA

binding (Y75A and Y128A) were indistinguishable from that of wild-type N4SSB, whereas N4SSB derivatives defective in transcriptional activation ( $\Delta 264$ -265; S260A and K264A;K265A) did not interact with RNAP (Fig. 2B). We conclude that N4SSB interacts with RNAP, most likely with the  $\beta'$  subunit.

In previous work, we identified a mutant of  $\beta'$  that is defective in N4 propagation (10, 11). This mutant,  $\beta'\Delta C$ , lacks the COOH-terminal 52 amino acids of  $\beta'$  (amino acids 1354 to 1407) and has in their place 23 nonnative amino acids (12). Here we reinvestigated the activity of  $\beta'\Delta C$  RNAP in transcription and found that  $\beta'\Delta C$  RNAP is defective in N4SSB-dependent transcription at N4 late promoters but not defective in N4SSB-independent transcription at the *rrb* P1 promoter (13, 14) (Fig. 3A). The simplest interpretation of these results is that the extreme COOH-terminal region of  $\beta'$  (amino acids 1354 to 1407) plays a specific role in, and may be the target for, transcriptional activation by N4SSB.

To identify directly the target for transcriptional activation by N4SSB, we performed site-specific protein-protein photo cross-linking (15, 16). We constructed an N4SSB derivative with a photoactivatable

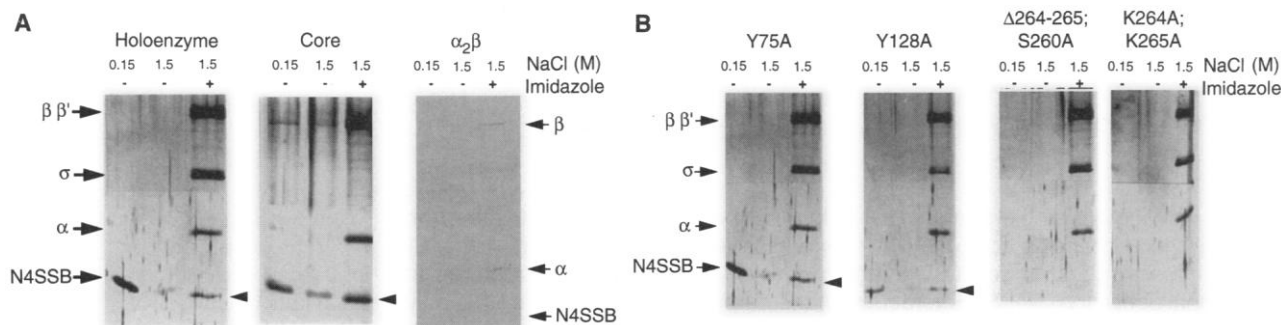


**Fig. 1.** Separation of transcriptional activation and ssDNA binding activities of N4SSB (6). (A) Transcriptional activation by N4SSB (WT, for wild type) and its derivatives. Top panel, in vivo; bottom panel, in vitro in the absence (left lane in each set) or presence of 1, 2, and 4  $\mu$ M N4SSB (increasing concentration indicated by the black wedge above the lanes). (B) Electrophoretic mobility gel shift experiment to assess ssDNA binding in the absence or presence of 0.1, 0.25, 0.5, and 0.75  $\mu$ g of N4SSB (indicated by the black wedge) (only bands corresponding to ssDNA-N4SSB complexes are shown). (C) N4 recombination and (D) N4 replication (circles, with IPTG induction of N4SSB synthesis; triangles, without IPTG induction).

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**Fig. 2.** Interaction of N4SSB with RNAP. **(A)** Interaction of wild-type N4SSB with RNAP holoenzyme (left), RNAP core (middle), and  $\alpha_2\beta$  (right) (8). **(B)** Interactions with RNAP holoenzyme of N4SSB mutants specifically defective in ssDNA binding (Y75A and Y128A) or specifically defective in transcriptional activation ( $\Delta 264-265$ ;S260A and K264A;K265A). Arrows indicate RNAP subunits and N4SSB; arrowheads indicate N4SSB retained by RNAP.

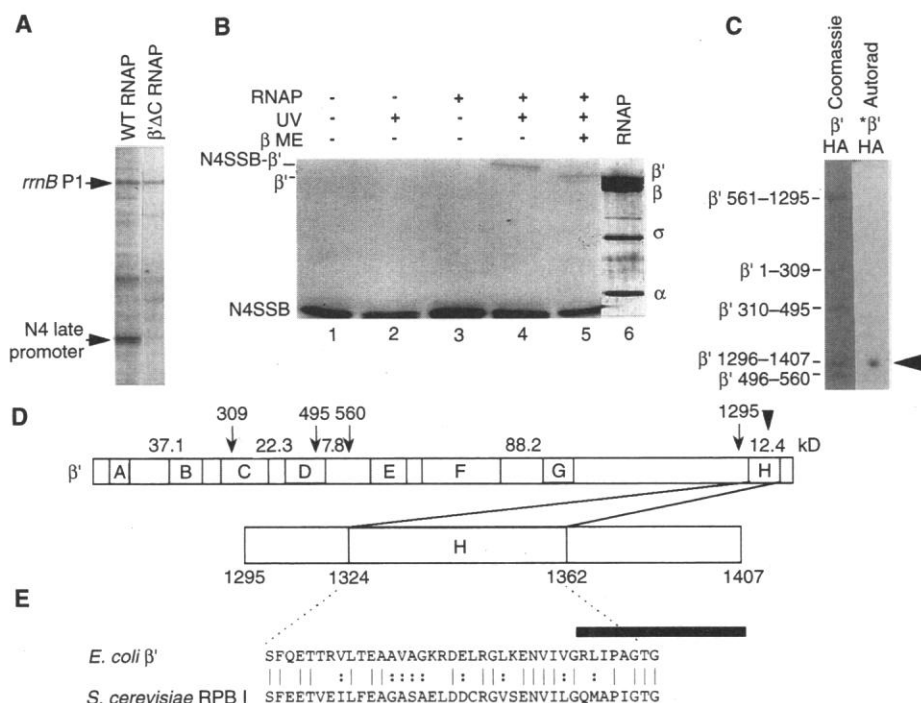
cross-linking agent incorporated at amino acid 251, which is adjacent to the genetically defined activating region (17). We then incubated the N4SSB derivative with RNAP to form the N4SSB-RNAP complex, irradiated the complex with ultraviolet (UV) light to initiate cross-linking, and determined the site in RNAP at which cross-linking occurred (17). To facilitate

identification of the cross-linked site, we used a photoactivatable cross-linking agent that contained a radiolabel and that was attached to N4SSB through a disulfide linkage (15-17). This permitted, after UV irradiation, cleavage of the cross-link and transfer of the radiolabel to the site at which cross-linking occurred. Cross-linking occurred exclusively within  $\beta'$  (Fig. 3B),

and limited proteolysis showed that cross-linking occurred exclusively within the region between amino acids 1296 to 1407 (Fig. 3C). We conclude that residues within the extreme COOH-terminal region of  $\beta'$  are in direct physical proximity to the activating region of N4SSB in the N4SSB-RNAP complex [within 16 Å of C $\alpha$  of amino acid 251 of N4SSB (15, 16)].

On the basis of the correspondence between the genetic results (which implicate amino acids 1354 to 1407 of  $\beta'$ ) and the photo cross-linking results (which implicate amino acids 1296 to 1407 of  $\beta'$ ), we conclude that transcriptional activation by N4SSB involves direct protein-protein interaction between the activating region of N4SSB and the extreme COOH-terminal region of  $\beta'$ .

Our results have two general implications. First, our results suggest that transcriptional activation may be possible in the absence of DNA binding—both direct DNA binding and indirect DNA binding with tethering by another DNA binding protein (18). Thus, the ssDNA binding activity of N4SSB is not required for transcriptional activation (Fig. 1), and N4SSB has no detectable dsDNA binding activity (2). (We cannot exclude the possibility that N4SSB has a hitherto undetected second DNA binding activity; for example, a low-affinity DNA binding activity or a cryptic DNA binding activity revealed only upon interaction with RNAP.) The proposal that N4SSB activates transcription without DNA binding raises the question of how N4SSB achieves specificity for target promoters. There are several possibilities: Specificity for target promoters might be achieved by “indirect readout” of the promoter sequence, with N4SSB preferentially recognizing a specific conformation that RNAP adopts at N4SSB-dependent promoters but not at N4SSB-independent promoters. Alternatively, the specificity might be achieved kinetically, with N4SSB affecting a step in transcription initiation that is



**Fig. 3.** Interaction of N4SSB with the RNAP  $\beta'$  subunit. **(A)** Effects of wild-type (WT) RNAP and  $\beta'\Delta C$  RNAP on N4SSB-dependent transcription at the N4 late promoter R and N4SSB-independent transcription at the *rrmB* P1 promoter (13). **(B)** Site-specific protein-protein cross-linking (17). Lane 4, photo cross-linking; lane 5, photo cross-linking followed by cleavage and radiolabel transfer; lanes 1 to 3, control reactions; lane 6, molecular size markers. **(C)** Proteolytic mapping. Gel slices containing  $\beta'$  from photo cross-linking followed by cleavage and radiolabel transfer (B, lane 5) were digested with hydroxylamine (HA) as described in (31), except that digestion was carried out for 6 hours at 45°C at pH 9.3. Left lane, Coomassie stained SDS-PAGE analysis; right lane, corresponding autoradiogram; arrowhead indicates cross-linking target. **(D)** HA cleavage sites on  $\beta'$ . Arrows indicate sites of HA cleavage, and numbers between the arrows indicate sizes of cleavage products. Boxed areas A to H indicate regions of homology in the largest subunits of prokaryotic and eukaryotic RNAP (22). **(E)** Sequences of *E. coli*  $\beta'$  and *Saccharomyces cerevisiae* region H. The bar above the  $\beta'$  sequence indicates the region deleted in  $\beta'\Delta C$  (12). Vertical bars indicate identical residues, and stacked dots indicate similar residues.

limiting at N4SSB-dependent promoters but not at N4SSB-independent promoters. Finally, N4SSB might have only a limited specificity for target promoters. (Although limited specificity would be unsuitable for most activators, it might be suitable for a lytic-viral activator, especially a late-phase, lytic-viral activator.) The proposal that N4SSB activates transcription without DNA binding also raises the question of how N4SSB achieves sufficient occupancy at target promoters. N4SSB is produced in very large amounts during N4 infection— $\sim 11,000$  molecules per cell, corresponding to  $10^{-5}$  M (2)—and, on the basis of our protein-protein interaction assays, appears to have a high affinity for RNAP. If, in fact, N4SSB activates transcription without DNA binding, it is likely to function not by facilitating the binding of RNAP to promoter DNA—at least not by “tethering” (19)—but, rather, by facilitating subsequent steps. Consistent with this view, preliminary results indicate that N4SSB has no effect on the binding of RNAP (20).

Second, our results raise the possibility that the COOH-terminal region of the largest subunit of RNAP may be an activation target in both prokaryotes and eukaryotes. There are now four known activation targets within prokaryotic RNAP: (i) the  $\alpha$  COOH-terminal domain (21), (ii) the  $\alpha$  NH<sub>2</sub>-terminal domain (16), (iii) the  $\sigma$  COOH-terminal region (22), and (iv) the  $\beta'$  subunit COOH-terminal region (Fig. 3). The target that we have identified in the  $\beta'$  COOH-terminal region contains part of region H (residues 1324 to 1362) (23) and a negatively charged segment following region H. Region H is conserved in sequence and the negatively charged segment is conserved in charge in the largest subunits of prokaryotic RNAP and eukaryotic RNAP II (23) (Fig. 3, D and E). In the largest subunit of eukaryotic RNAP II, region H and the negatively charged segment are immediately followed by 17 to 52 copies of a tandemly repeated heptapeptide motif (CTD) (24). Structural studies of RNAP II show that the COOH-terminal end of region H, the negatively charged segment, and CTD are located at the trailing edge of RNAP,  $\sim 80$  Å from the RNAP active site (25). Several lines of evidence suggest that region H, the negatively charged segment, and CTD interact with each other and are involved in transcriptional activation. First, substitutions within region H suppress effects of deletions within CTD (24, 26, 27). Second, region H and the negatively charged segment interact with transcription factors, including TATA-binding protein and TFIIB (28). Third, CTD is required for the response to several activators (24, 26, 29). Finally, the phosphorylation state of CTD is

important for transcription and is affected by activators (23, 30). It will be of interest to determine whether the interaction of activators with the COOH-terminal regions of the largest subunits of prokaryotic and eukaryotic RNA polymerases have similar mechanistic consequences.

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- In Y75A, N4SSB residue Tyr (Y) at position 75 was substituted with Ala (A); in Y128A, residue Tyr<sup>128</sup> was substituted with Ala; in  $\Delta 264-265$ ; S260A, residues 264 and 265 were deleted and Ser (S) at position 260 was substituted with Ala; and in K264A; K265A, residues Lys (K) at positions 264 and 265 were substituted with Ala.
- Strain W3350pcnB(DE3)/pLyse containing the N4SSB expression vector pMC6 (3) or a derivative was infected with N4am7, a phage deficient in N4SSB activity (2), and analyzed for transcriptional activation at the N4 late promoter GF180 (primer extension), recombination (production of wild-type phage), or replication (<sup>3</sup>H]thymidine incorporation), before or after induction with isopropyl-thio- $\beta$ -D-galactoside (IPTG) (1, 3). The ability of N4SSB and its derivatives to activate transcription in vitro was measured in run-off transcription experiments with an 800-base pair (bp) DNA fragment containing the N4 late promoter R (which uses three start sites), RNAP, and increasing amounts of N4SSB (1). The ssDNA binding was assayed in electrophoretic mobility shift experiments (3). N4SSB and its derivatives were purified as in (7).
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- Thr<sup>251</sup>, which is not essential for activation as determined by deletion analysis (4), was replaced by Cys, and the two preexisting Cys residues within N4SSB (3) were replaced by Ser residues. The resulting N4SSB derivative was fully functional in transcriptional activation, both before and after derivatization with the photo cross-linking agent. Derivatization with [<sup>125</sup>I]-[S-[2-(4-azidosalicylamido)ethylthio]-2-thiopyridine] (<sup>125</sup>I-AET), photo cross-linking, cleavage after photo cross-linking, and radiolabel transfer were performed as in (15, 16). Reaction mixtures for photo cross-linking contained 250 nM N4SSB derivative and 125 nM RNAP (Epicentre Biotechnologies, Madison, WI). Control experiments with wild-type N4SSB with the cross-linking agent incorporated at C84 and C86 yielded no N4SSB-RNAP cross-linking.
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