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# Determinants of RNA polymerase α subunit for interaction with β, β′, and σ subunits: Hydroxyl-radical protein footprinting

**(protein–protein interaction/protein–ligand interaction/transcription/epitope mapping)**

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ABSTRACT *Escherichia coli* **RNA polymerase (RNAP) α subunit serves as the initiator for RNAP assembly, which proceeds according to the pathway $2\alpha \rightarrow \alpha_2 \rightarrow \alpha_2\beta \rightarrow \alpha_2\beta\beta' \rightarrow \alpha_2\beta\beta'\sigma$. In this work, we have used hydroxyl-radical protein footprinting to define determinants of α for interaction with β, β′, and σ. Our results indicate that amino acids 30–75 of α are protected from hydroxyl-radical-mediated proteolysis upon interaction with β (i.e., in $\alpha_2\beta$, $\alpha_2\beta\beta'$, and $\alpha_2\beta\beta'\sigma$), and amino acids 175–210 of α are protected from hydroxyl-radical-mediated proteolysis upon interaction with β′ (i.e., in $\alpha_2\beta\beta'$ and $\alpha_2\beta\beta'\sigma$). The protected regions are conserved in the α homologs of prokaryotic, eukaryotic, archaeal, and chloroplast RNAPs and contain sites of substitutions that affect RNAP assembly. We conclude that the protected regions define determinants of α for direct functional interaction with β and β′. The observed maximal magnitude of protection upon interaction with β and the observed maximal magnitude of protection upon interaction with β′ both correspond to the expected value for complete protection of one of the two α protomers of RNAP (i.e., 50% protection). We propose that only one of the two α protomers of RNAP interacts with β and that only one of the two α protomers of RNAP interacts with β′.**

*Escherichia coli* RNA polymerase holoenzyme (RNAP) has subunit composition $\alpha_2\beta\beta'\sigma$ (for review, see ref. 1). RNAP α, the smallest subunit (329 amino acids), performs at least three critical functions:

(*i*) RNAP α serves as the initiator for RNAP assembly, which proceeds according to the pathway $2\alpha \rightarrow \alpha_2 \rightarrow \alpha_2\beta \rightarrow \alpha_2\beta\beta' \rightarrow \alpha_2\beta\beta'\sigma$ (for review, see ref. 2).

(*ii*) RNAP α participates in promoter recognition, making direct sequence-specific α–DNA interactions with promoter upstream elements (ref. 3; for review, see refs. 4 and 5).

(*iii*) RNAP α participates in transcriptional activation, repression, and elongation, making direct protein–protein interactions with activators, repressors, and elongation factors (refs. 6–9; for review, see refs. 4 and 5).

RNAP α consists of two independently folded domains: an N-terminal domain required for RNAP assembly (amino acids 8–235) and a C-terminal domain required for interactions with promoter upstream elements, activators, repressors, and elongation factors (amino acids 249–329) (refs. 10 and 11; for review, see refs. 4 and 5).

The α N-terminal domain, by itself, is able to dimerize and to be assembled into RNAP (6, 12–14). Therefore, the α N-terminal domain must contain determinants for dimerization, for interaction with β, and, possibly, for interactions with β′ and σ. Amino acid substitutions and insertions at amino acids 45, 48, and 80 of α block formation of $\alpha_2\beta$ but do not block formation of $\alpha_2$, indicating that these amino acids may be part of the determinant for interaction with β (15–18). Analogously, amino acid substitutions and insertions at amino acids 86, 173, 180, and 200 of α block formation of $\alpha_2\beta\beta'$ but do not block formation of $\alpha_2\beta$, indicating that these amino acids may be part of the determinant for interaction with β′ (17, 18).

The α N-terminal domain (but not the α C-terminal domain) is conserved in the α homologs of prokaryotic, eukaryotic, archaeal, and chloroplast RNAP (5). An amino acid substitution at the position equivalent to amino acid 40 in the α homolog of yeast RNAP II (RPB3) blocks interaction with the β homolog (RPB2) and results in a conditional-lethal growth phenotype (19), and amino acid substitutions at the positions equivalent to amino acids 40, 41, 44, 45, 54, and 59 in the α homolog of yeast RNAP I and III (RPAC40) result in lethal or conditional-lethal growth phenotypes (20), suggesting that determinants for RNAP assembly may be conserved.

The genetic and sequence-comparison results, by themselves, do not distinguish between amino acids of α involved directly in interactions with β and β′ and amino acids of α involved solely in maintaining the proper conformation of α. In this work, we have used a biochemical method—i.e., hydroxyl-radical protein footprinting—to define determinants of α for interaction with β, β′, and σ.

Our procedure for hydroxyl-radical protein footprinting has three steps: (*i*) we $^{33}$P-end-label the protein of interest [using an introduced recognition site for heart-muscle protein kinase (HMPK); methods in ref. 21]; (*ii*) in parallel reactions, we carry out hydroxyl-radical-mediated cleavage of the $^{33}$P-end-labeled protein and of the $^{33}$P-end-labeled protein in complex with a ligand (methods in refs. 22 and 23; description of cleavage chemistry in refs. 24–27); and (*iii*) we analyze the cleavage products by denaturing PAGE followed by PhosphorImager analysis. Binding of the ligand decreases polypeptide-backbone solvent accessibility at residues it contacts, protecting against hydroxyl-radical-mediated cleavage at residues it contacts and, therefore, resulting in a gap in the "ladder" of cleavage products. The location of the ligand binding site can be read out directly from the location of the gap in the ladder of cleavage products.

## MATERIALS AND METHODS

**Preparation of $\alpha^*_2$, $\alpha^*_2\beta$, $\alpha^*_2\beta\beta'$, and $\alpha^*_2\beta\beta'\sigma$.** pHTT7f1-NHKα, which encodes an N-terminally hexahistidine-tagged, N-terminally HMPK-tagged α derivative (MHHHHHHR-RASVA, followed by amino acids 2–329 of α) under control of the bacteriophage T7 gene *10* promoter, was constructed from pHTT7f1-NHα (28) using site-directed mutagenesis (29). The α derivative was overproduced in *E. coli* (procedures in ref. 28),

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Abbreviations: RNAP, RNA polymerase; HMPK, heart-muscle protein kinase.

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was purified using nondenaturing batch-mode metal-ion-affinity chromatography on $Ni^{++}$-NTA-agarose (Qiagen; procedures in ref. 28), and was $^{33}$P-end-labeled in a reaction containing (300 μl) 60 μM α derivative, 500 units of HMPK catalytic subunit (Sigma, catalog no. P2645), 1 μM [$\gamma^{33}$P]ATP (70 Bq/fmol; Amersham), 10 mM Tris·HCl (pH 7.9), 150 mM KCl, and 6 mM dithiothreitol (1 h at 37°C).

$\alpha_2^*\beta$, $\alpha_2^*\beta\beta'$, and $\alpha_2^*\beta\beta'\sigma$ were prepared by addition of excess β, β and β′, or β, β′, or $\sigma^{70}$, under denaturing conditions, followed by dialysis into nondenaturing conditions and batch-mode metal-ion-affinity chromatography (procedures essentially as in ref. 30), followed by gel-filtration chromatography on Superose-6 (Pharmacia) (procedures as in ref. 31 except that dithiothreitol and glycerol were omitted from the running buffer).

**Preparation of $\alpha_2^{*C}$, $\alpha_2^{*C}\beta$, $\alpha_2^{*C}\beta\beta'$, and $\alpha_2^{*C}\beta\beta'\sigma$.** pHTT7f1-NHαK, which encodes an N-terminally hexahistidine-tagged, C-terminally HMPK-tagged α derivative (MHHHHHH, followed by amino acids 2–329 of α, followed by RRASVA) under control of the bacteriophage T7 gene 10 promoter, was constructed from pHTT7f1-NHα (28) using site-directed mutagenesis (29). The α derivative was overproduced, purified, $^{33}$P-end-labeled, and assembled into higher-order complexes by the procedures of the preceding section.

**Hydroxyl-Radical Protein Cleavage.** Reaction mixtures contained (10 μl): 4 μM $\alpha_2^*$, $\alpha_2^*\beta$, $\alpha_2^*\beta\beta'$, $\alpha_2^*\beta\beta'\sigma$, $\alpha_2^{*C}$, $\alpha_2^{*C}\beta$, $\alpha_2^{*C}\beta\beta'$, or $\alpha_2^{*C}\beta\beta'\sigma$ (1 Bq/fmol), 2 mM EDTA, 1 mM $(NH_4)_2Fe(II)(SO_4)_2$, 1 mM $H_2O_2$, 20 mM sodium ascorbate, 10 mM Mops·NaOH (pH 7.2), 5 mM Tris·HCl, 125 ml NaCl, 2 μM $ZnCl_2$, and 1% glycerol. Reactions were initiated by addition of EDTA and $(NH_4)_2Fe(II)(SO_4)_2$ [as a freshly prepared solution containing 20 mM EDTA and 10 mM $(NH_4)_2Fe(II)(SO_4)_2$], $H_2O_2$, and sodium ascorbate. Reactions were terminated after 30 min at 25°C by addition of 5 μl 3× loading buffer [150 mM Tris·HCl, pH 7.9/36% glycerol/12% SDS/6% 2-mercaptoethanol/0.01% bromophenol blue], and products were analyzed by tricine SDS/PAGE (22, 32) followed by PhosphorImager analysis (Molecular Dynamics model 425B PhosphorImager).

**Residue-Specific Protein Cleavage.** Reaction mixtures for methionine-specific cleavage contained (20 μl; pH adjusted to 2 with 1 M HCl): 3 μM $\alpha_2^*$ or $\alpha_2^{*C}$ (1 Bq/pmol), 500 mM CNBr, and 0.4% SDS. Reactions were terminated after 20 min at 25°C by lyophilization and addition of 50 μl 1× loading buffer. Reaction mixtures for lysine- and glutamic acid-specific cleavage contained (60 μl) 30 nM $\alpha_2^*$ or $\alpha_2^{*C}$ (1 Bq/pmol), 0.2 μg of endoproteinase Lys-C (Promega) or 0.5 μg of endoproteinase Glu-C (Sigma), 8 M urea, and 50 mM Tris·HCl (pH 9.0). Reactions were terminated after 15 min at 25°C by addition of 30 μl of 3× loading buffer followed by boiling.

**Data Analysis.** Phosphorimager intensities were integrated across full lane widths and plotted versus electrophoretic mobilities using IMAGEQUANT (Molecular Dynamics). Intensity plots were aligned, correcting for lane-to-lane gel distortions, using ALIGN [available on request; written in BASIC and running under LABWINDOWS (National Instruments)]. Aligned intensity plots were imported into SIGMAPLOT (Jandel), backgrounds were subtracted, cleavage and gel-loading efficiencies were normalized, data from multiple lanes were averaged, electrophoretic mobilities were converted into residue numbers (residue numbers as in wild-type α), and difference plots were calculated, using DIFFPLOT (available on request; written in SIGMAPLOT transform language). Cleavage and gel-loading efficiencies were normalized using, for each pair of aligned intensity plots, the modal ratio from a histogram of pixel-by-pixel intensity ratios (excluding the portions of the aligned intensity plots corresponding to uncleaved full-length protein). This method of normalization assumes that a significant fraction of intensities is unaffected by interaction with the ligand of interest. This assumption appears to be valid for interaction of $\alpha_2^*$ and $\alpha_2^{*C}$ with β, β′, and σ.

During electrophoresis, cleavage products shorter than ≈40 amino acids were lost, and cleavage products 90–100% of full-length were not well resolved (Fig. 1). Therefore, difference plots were calculated excluding the extreme N and C termini [amino acids 1–20 and 285–329 in experiments using $\alpha_2^*$ (Fig. 2); amino acids 1–40 and 295–329 in experiments using $\alpha_2^{*C}$ (Fig. 3)].

## RESULTS

For this work, we have constructed a plasmid encoding an α derivative having an N-terminal HMPK recognition site and hexahistidine affinity tag, and we have overproduced, purified, and $^{33}$P-end-labeled the α derivative. Starting with the $^{33}$P-end-labeled α derivative ($\alpha_2^*$), we formed homogeneous $\alpha_2^*\beta$, $\alpha_2^*\beta\beta'$, and $\alpha_2^*\beta\beta'\sigma$ complexes by addition of excess β, excess β and β′, or excess β, β′, and σ, respectively, followed by metal-ion-affinity chromatography (cf. refs. 28 and 30). For each complex, we then performed hydroxyl-radical-mediated cleavage and compared the cleavage pattern to that with $\alpha_2^*$ alone (Fig. 1).

To facilitate objective comparison, we developed and used software for quantitative data acquisition and analysis. The software aligned bands, subtracted backgrounds, corrected for cleavage and loading efficiencies, and averaged data from multiple lanes. The software then prepared "difference plots," comparing averaged data for each complex ($\alpha_2^*\beta$, $\alpha_2^*\beta\beta'$, and $\alpha_2^*\beta\beta'\sigma$) to averaged data for $\alpha_2^*$ alone (Fig. 2). The resulting difference plots exhibited good reproducibility and low background noise (Fig. 2*A*). Essentially identical difference plots were obtained in experiments using four preparations of $\alpha_2^*$ (data not shown) and also in parallel experiments using an α derivative radiolabeled at its C terminus ($\alpha_2^{*C}$; Fig. 3).

**Determinants of α for Interaction with β: $\alpha_2^*\beta$ versus $\alpha_2^*$.** Fig. 2*B* compares the cleavage pattern with the $\alpha_2^*\beta$ complex to that with $\alpha_2^*$ alone. Addition of β results in protection of the region corresponding to amino acids 30–75 of α. The protection exhibits a reproducible fine structure, with strongest protection at amino acids 30–55 and 65–75. The maximum magnitude of protection is 52 ± 3% (normalized difference of −1.1 ± 0.1 in Figs. 2*B Right* and 3*B Right*); within error, this is equal to the expected value for complete protection of one of the two α* subunits of $\alpha_2^*\beta$ (50%). We conclude that addition of β reduces polypeptide-backbone solvent accessibility in the region corresponding to amino acids 30–75 of α, and we propose that β directly contacts this region.

**Determinants of α for Interaction with β′: $\alpha_2^*\beta\beta'$ versus $\alpha_2^*$.** Fig. 2*C* compares the cleavage pattern with the $\alpha_2^*\beta\beta'$ complex to that with $\alpha_2^*$ alone. There are two protected regions. The first protected region corresponds to the region protected by β in the $\alpha_2^*\beta$ complex (cf. Fig. 2 *B* and *C*). Neither the boundaries nor the fine structure of the protected region change. We conclude that addition of β′ does not substantially alter the interactions made by β. The second protected region is observed only upon addition of β′ (cf. Fig. 2 *B* and *C*). The second protected region corresponds to amino acids 175–210 of α and exhibits a reproducible fine structure, with strongest protection at amino acids 175–185 and 195–210. The maximum magnitude of the second protection is 47 ± 7% (normalized difference of −0.9 ± 0.3 in Figs. 2*C Right* and 3*C Right*); within error, this is equal to the expected value for complete protection of one of the two α* subunits of $\alpha_2^*\beta\beta'$ (50%). We conclude that addition of β′ reduces polypeptide-backbone solvent accessibility in the region corresponding to amino acids 175–210 of α, and we propose that β′ directly contacts this region.

**Determinants of α for Interaction with σ: $\alpha_2^*\beta\beta'\sigma$ versus $\alpha_2^*$.** Fig. 2*D* compares the cleavage pattern with the $\alpha_2^*\beta\beta'\sigma$

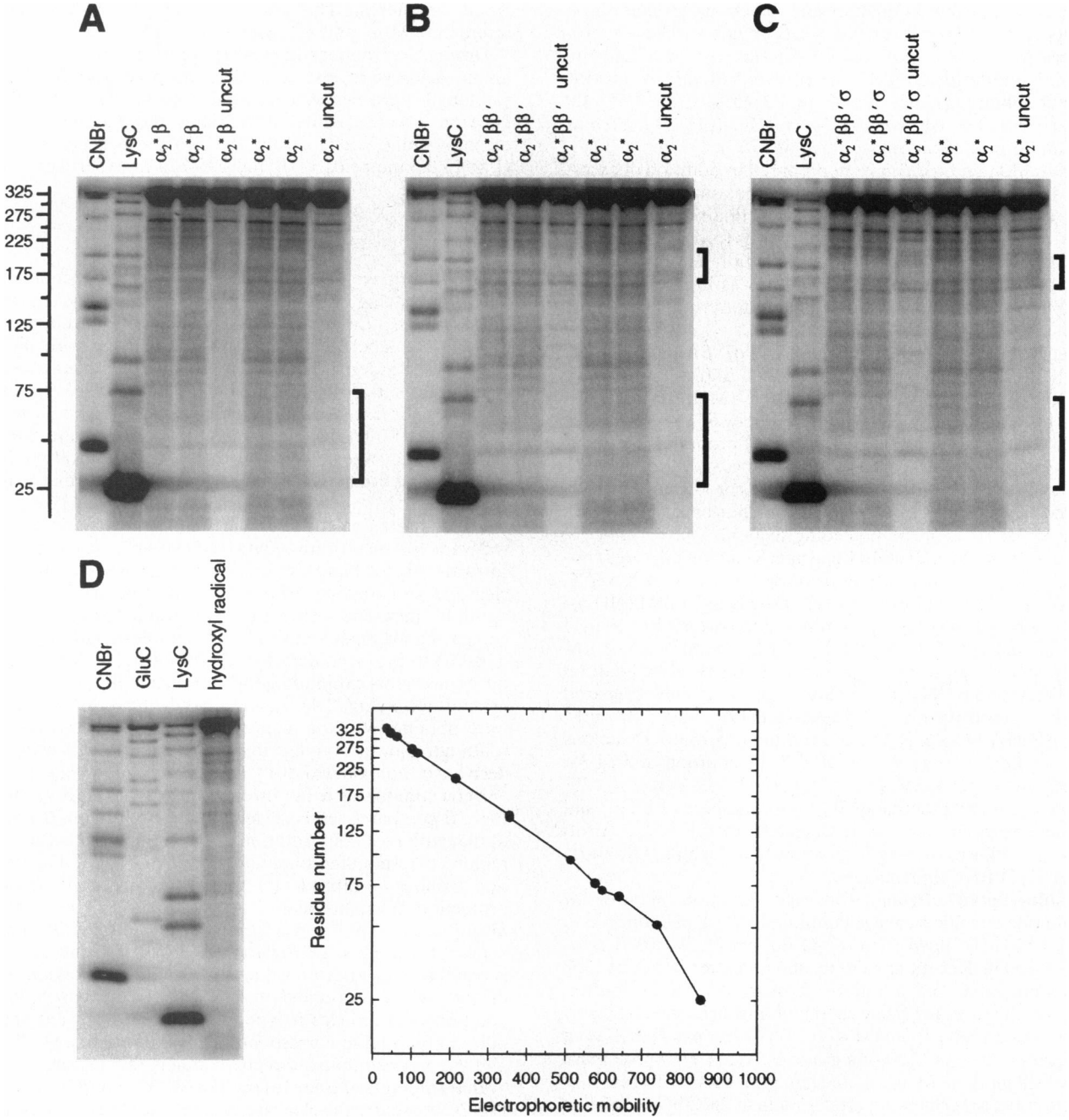


FIG. 1. Hydroxyl-radical protein footprinting: data for $\alpha_2^*\beta$ (*A*), $\alpha_2^*\beta\beta'$ (*B*), and $\alpha_2\beta\beta'\sigma$ (*C*). Molecular weight markers were generated by residue-specific cleavage of denatured $\alpha_2^*$ under single-hit conditions using CNBr, endoproteinase Lys-C, and endoproteinase Glu-C [specific for methionine, lysine, and glutamic acid, respectively (33)] (*D Left*). Hydroxyl-radical cleavage sites were assigned by interpolation (*D Right*).

complex to that with $\alpha_2^*$ alone. There are two protected regions. The first corresponds to the region protected by $\beta$ in the $\alpha_2^*\beta$ and $\alpha_2^*\beta\beta'$ complexes, and the second corresponds to the region protected by $\beta'$ in the $\alpha_2^*\beta\beta'$ complex (cf. Fig. 2 *B–D*). For each, neither the boundaries nor the fine structure change. We conclude that addition of $\sigma$ does not substantially alter the interactions made by $\beta$ and $\beta'$. There are no additional protected regions observed only upon addition of $\sigma$ (cf. Fig. 2 *C* and *D*). Therefore, we tentatively conclude, in agreement with ref. 23, that $\sigma$ does not contact $\alpha$. We emphasize that this conclusion must be considered tentative, since our analysis excludes the extreme N and C termini of $\alpha$ and since our threshold for significance of effects may exclude weak contacts. McMahan and Burgess (34) have reported that $\sigma$ can be crosslinked to $\alpha$ within RNAP. However, the crosslinking results indicate proximity—not necessarily direct contact (34).

## DISCUSSION

Our results indicate that amino acids 30–75 of $\alpha$ are protected from hydroxyl-radical-mediated proteolysis upon interaction with $\beta$ (Figs. 2 *B–D*, 3 *B–D*, and 4*A*), and amino acids 175–210 of $\alpha$ are protected from hydroxyl-radical-mediated proteolysis upon interaction with $\beta'$ (Figs. 2 *C* and *D*, 3 *C* and *D*, and 4*A*).

Five regions of $\alpha$ are conserved in $\alpha$ homologs of prokaryotic, eukaryotic, archaeal, and chloroplast RNAP: regions A, B, C, D, and E (Fig. 4*B*). Strikingly, the segments of $\alpha$ most strongly protected by $\beta$ (amino acids 30–55 and 65–75) correspond nearly exactly to regions A and B, and the segments of $\alpha$ most strongly protected by $\beta'$ (amino acids 175–185 and 195–210) correspond nearly exactly to regions C and D (cf. Fig. 4 *A* and *B*). We propose that, in all organisms, regions A and B constitute the determinant for interaction with the $\beta$ ho-

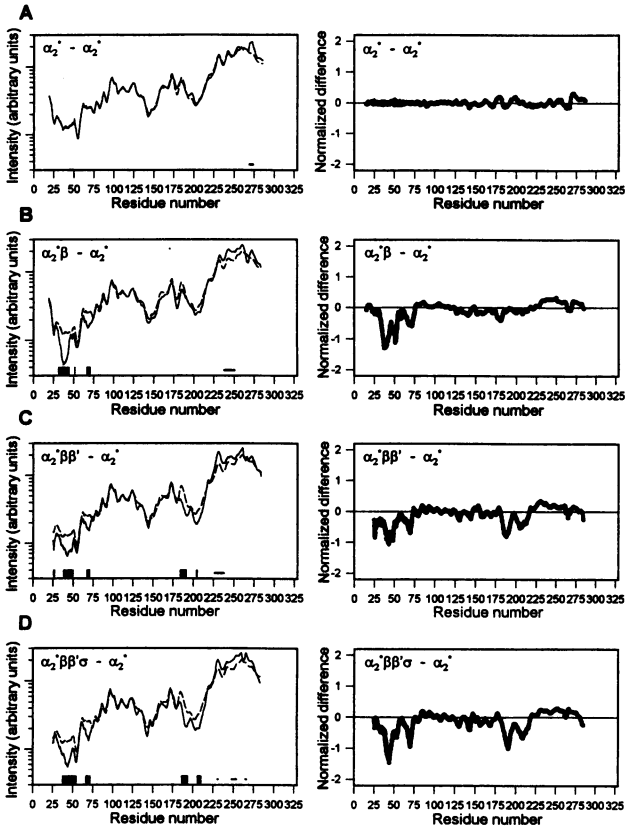


FIG. 2. Hydroxyl-radical protein footprinting: comparison of $\alpha_2^*$ versus $\alpha_2^*$ (averaged data for 6 lanes versus averaged data for six lanes) (*A*), $\alpha_2^*\beta$ versus $\alpha_2^*$ (*B*), $\alpha_2^*\beta\beta'$ versus $\alpha_2^*$ (*C*), and $\alpha_2^*\beta\beta'\sigma$ versus $\alpha_2^*$ (*D*). (*Left*) Corrected PhosphorImager intensities for the complex under study (solid line) and $\alpha_2^*$ (dashed line). Positions at which the corrected PhosphorImager intensities for the complex under study and $\alpha_2^*$ differ by at least 33% are marked with vertical ticks (decreases) or points (increases). (*Right*) Difference plots. Difference plots show $(I - I_{\alpha 2^*})/I$ versus residue number, where $I$ is the corrected intensity for the complex under study, and $I_{\alpha 2^*}$ is the corrected intensity for $\alpha_2^*$. Complete protection of one of the two $\alpha$ protomers in the complex under study would result in a value of $-1$.

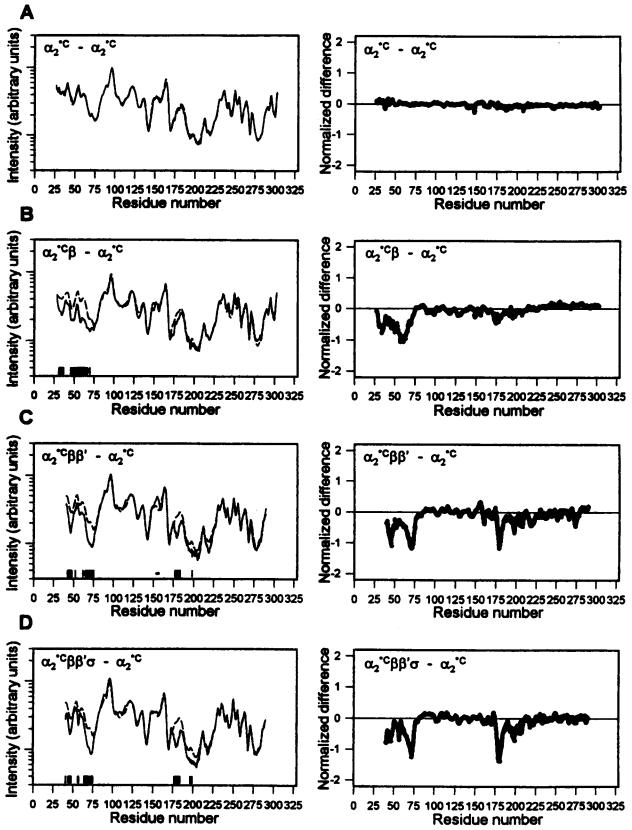


FIG. 3. Hydroxyl-radical protein footprinting: comparison of $\alpha_2^{*C}$ versus $\alpha_2^{*C}$ (averaged data for six lanes versus averaged data for six lanes) (*A*), $\alpha_2^{*C}\beta$ versus $\alpha_2^{*C}$ (*B*), $\alpha_2^{*C}\beta\beta'$ versus $\alpha_2^{*C}$ (*C*), and $\alpha_2^{*C}\beta\beta'\sigma$ versus $\alpha_2^{*C}$ (*D*). The difference plots for the C terminally radiolabeled $\alpha$ derivative $\alpha_2^{*C}$ are identical within error to the difference plots for the N-terminally radiolabeled $\alpha$ derivative $\alpha_2^*$.

molog, and regions C and D constitute the determinant for interaction with $\beta'$ homolog. [The remaining conserved region, region E, contains a potential leucine zipper (35). We speculate that region E constitutes the primary determinant for dimerization.]

Substitution of amino acid 45, 48, or 80 of $\alpha$ blocks interaction with $\beta$ (refs. 15–18; see also refs. 19 and 20), and substitution of amino acid 86, 173, 180, or 200 of $\alpha$ blocks interaction with $\beta'$ (17, 18). The segments of $\alpha$ most strongly protected by $\beta$ and $\beta'$ contain, or are immediately adjacent to, all except one of these sites.

The correspondence between the hydroxyl-radical protein footprinting results and the sequence-comparison and genetic results strongly suggests that the regions of $\alpha$ protected by $\beta$ and $\beta'$ are involved in direct, functional interaction with $\beta$ and $\beta'$.

Experiments with monoclonal antibodies directed against $\alpha$ indicate that the two $\alpha$ protomers of RNAP occupy nonidentical environments (36). The observed maximal magnitude of protection upon interaction with $\beta$ and the observed maximal magnitude of protection upon interaction with $\beta'$ both correspond to the expected value for complete protection of one of the two $\alpha$ protomers of RNAP (50% protection; normalized difference of $-1$ in Figs. 2 *B–D Right* and 3 *B–D Right*). We propose that only one of the two $\alpha$ protomers of RNAP interacts with $\beta$ and that only one of the two $\alpha$ protomers of

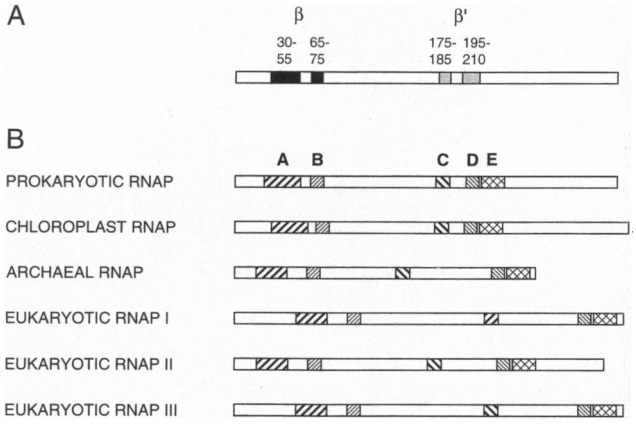


FIG. 4. (*A*) Summary of results. (*B*) Comparison of sequences of $\alpha$ homologs in prokaryotic RNAP (*E. coli* $\alpha$), chloroplast RNAP (maize $\alpha$), eukaryotic RNAP I (*Saccharomyces cerevisiae* RPAC40), eukaryotic RNAP II (*S. cerevisiae* RPB3), eukaryotic RNAP III (*S. cerevisiae* RPAC40), and archaeal RNAP (*Haloarcula marismortui* Rp$\alpha$) (citations in ref. 5). Five regions are conserved in all $\alpha$ homologs: A–E. Prokaryotic and chloroplast $\alpha$ homologs contain a C-terminal region involved in promoter recognition and transcriptional regulation (3–9); this region is not conserved in eukaryotic and archaeal $\alpha$ homologs.

and an $\alpha$ derivative defective in interaction with both $\beta$ and $\beta'$) supports this proposal and further suggests that it is the same $\alpha$ protomer that interacts with $\beta$ and $\beta'$ (W. Niu and R.H.E., unpublished data).

The reagents and procedures of this report should be generalizable to analysis of interactions of $\alpha$ with fragments of $\beta$ and $\beta'$ (37) and to analysis of interactions of $\alpha$ with DNA, activators, repressors, and elongation factors (3–9). In principle, the procedures of this report should be generalizable to analysis of any multiprotein or nucleoprotein complex.

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