Mapping of Subunit-Subunit Contact Surfaces on the $\beta'$ Subunit of Escherichia coli RNA Polymerase*

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The RNA polymerase core enzyme of Escherichia coli with the catalytic activity of RNA polymerization is assembled sequentially under the order: $2\alpha \rightarrow \alpha_2 \rightarrow \alpha_\beta \rightarrow \alpha_\beta \beta'$. The core enzyme gains the activities of promoter recognition and transcription initiation after binding the $\sigma$ subunit. The subunit-subunit contact surfaces of $\beta'$ subunit (1407 residues) were analyzed by testing complex formation between various $\beta'$ fragments and either the $\alpha_\beta$ complex or the $\sigma^{70}$ subunit. Results indicate that two regions, one central region between residues 515 and 842 and the other COOH-terminal proximal region of the $\sigma^{70}$ subunit, was determined by x-ray crystallography (9), whereas the carboxyl (COOH)-terminal domain is involved in transcription regulation through direct interactions (3–5), whereas the carboxyl (COOH)-terminal domain is involved in transcription regulation through direct interactions (3–5), whereas the carboxyl (COOH)-terminal domain is involved in transcription regulation through direct interactions (3–5), whereas the carboxyl (COOH)-terminal domain is involved in transcription regulation through direct interactions (3–5), whereas the carboxyl (COOH)-terminal domain is involved in transcription regulation through direct interactions (3–5).

The subunit-subunit contact surfaces of $\beta'$ subunit (1407 residues) were analyzed by testing complex formation between various $\beta'$ fragments and either the $\alpha_\beta$ complex or the $\sigma^{70}$ subunit. Results indicate that two regions, one central region between residues 515 and 842 and the other COOH-terminal proximal region downstream from residue 1141, are involved in binding the $\alpha_\beta$ complex; and the NH2-terminal proximal region between residues 201 and 345 plays a major role in binding the $\sigma^{70}$ subunit. However, both $\alpha_\beta$ binding sites have weak activity of the $\sigma^{70}$ subunit; likewise, the $\sigma^{70}$ subunit-contact surface has weak binding activity of the $\alpha_\beta$ complex. The sites involved in the catalytic function of RNA polymerization are all located within two spacer regions sandwiched between these three subunit-subunit contact surfaces.

The RNA polymerase holoenzyme of Escherichia coli is composed of the core enzyme with the subunit composition of $\alpha_\beta \beta'$ and one of seven different species of the $\sigma$ subunit (for review, see Ref. 1). The core enzyme carries the catalytic activities for RNA polymerization, but the $\sigma$ subunit is required for promoter recognition and transcription initiation from the promoters. The core enzyme is assembled sequentially both in vitro and in vivo under the order: $2\alpha \rightarrow \alpha_2 \rightarrow \alpha_\beta \rightarrow \alpha_\beta \beta'$ (premature core) → E (active core) (for review, see Ref. 2).

Genetic and biochemical studies indicated that the subunit-subunit contact sites on $\alpha$ including the sites for $\alpha$ dimerization and the contact sites with the $\beta$ and $\beta'$ subunits are all located within the amino (NH2)-terminal domain down to residue 235 (3–5), whereas the carboxyl (COOH)-terminal domain is involved in transcription regulation through direct interactions with class I (or $\alpha$ contact) transcription factors and DNA UP elements (1, 5–7). The NMR structure has been determined for the $\alpha$ COOH-terminal domain (8); the structure of the $\alpha$ NH2-terminal domain was determined by x-ray crystallography (9), and the two domains are connected by a long flexible linker (10). Detailed mapping of the $\alpha_\alpha$, $\alpha_\beta$, and $\alpha_\beta'$ contact sites on the $\alpha$ subunit have been carried out by making a number of contact-defective $\alpha$ mutants with deletion, insertion, and Ala substitution mutations (11–14) or by mapping the cleavage sites in $\alpha$ by a chemical protease conjugated at various positions of the $\alpha$ subunit (15).

On the contrary, relatively little is known on the subunit-subunit contact sites on the two large subunits, $\beta$ and $\beta'$. The mapping of $\alpha$ subunit contact sites on the $\beta$ subunit was carried out using two approaches: analysis of the proteolytic cleavage pattern of the unassembled free $\beta$ subunit and the intermediate subassembly $\alpha_\beta$ complex (16, 17), and analysis of complex formation between various $\beta$ fragments and the hexahistidine (His6)-tagged $\alpha$ subunit or between various His6-tagged $\beta$ fragments and the intact $\alpha$ subunit (17). The results altogether indicate that the primary and tight contact site of the $\alpha$ subunit is located in the central portion of the $\beta$ polypeptide (16, 17); but in addition, the COOH-terminal proximal region is needed as the secondary and stable regulatory site for either efficient binding of the $\alpha$ subunit or stabilization of $\alpha_\beta$ contact (17). All of the $\beta$ fragment-$\alpha$ binary complexes isolated were able to bind the $\beta'$ subunit, leading to formation of pseudo-core complexes, suggesting that the $\beta'$ subunit contact site(s) on the $\beta$ subunit is(are) located near the $\alpha$ contact sites. In addition to contact with two core subunits, $\alpha$ and $\beta'$, the $\beta$ subunit seems to interact with the $\sigma$ subunit at its COOH-terminal proximal region (18).

At present, our knowledge of the location of subunit-subunit contact sites on the $\beta'$ subunit is limited. The isolated $\beta'$ subunit is able to form binary complexes with the $\sigma^{70}$ subunit (19). The contact site of the $\sigma^{70}$ subunit on $\beta'$ has been analyzed using different methods. (i) The assay of $\sigma^{70}$ subunit binding to pseudo-core enzymes containing various $\beta'$ fragments indicates that the deletion mutant $\beta'$ lacking amino acid residues 201–477 is unable to bind to the $\sigma^{70}$ subunit (20). (ii) A short $\beta'$ fragment containing residues 260–309 forms complexes with $\sigma^{70}$, as measured by co-immobilization assay using His6-tagged $\beta'$ fragments (21). (iii) Essentially the same NH2-terminal proximal region of $\beta'$ is cleaved by a chemical protease conjugated to $\sigma^{70}$ (22). The isolated $\beta$ and $\beta'$ subunits do not form stable binary complexes under isolated states (19), but these two subunits contact each other in the assembled RNA polymerase, suggesting that the $\alpha$ subunit plays a role in supporting the contact between the $\beta$ and $\beta'$ subunits. In the assembled core enzyme, one $\alpha$ subunit contacts the $\beta$ subunit while the other $\alpha$ makes direct contact with the $\beta'$ subunit (23).

In this study, we tried to map the subunit-subunit contact sites on the $\beta'$ subunit with not only the $\sigma^{70}$ subunit but also the $\alpha_\beta$ complex using two approaches. First, limited proteolysis by trypsin was carried out to generate $\beta'$ fragments for analysis of protease-sensitive sites exposed on the unassembled free $\beta'$ subunit. To identify the subunit-subunit contact sites on $\beta'$, the tryptic fragments were tested for complex formation with preformed $\alpha_\beta$ complex or purified $\sigma^{70}$ subunit.
For detailed mapping, various β' fragments with or without the His6 tag were expressed, purified, and analyzed for complex formation with the αβ complex and the σ70 subunit. Results indicate that the NH2-terminal proximal region between residues 201 and 345 is central for binding of the σ70 subunit, and at least two regions between 515 and 842, and 1141 and 1407 are involved in binding of the αβ complex.

EXPERIMENTAL PROCEDURES

Construction of Expression Plasmids—Plasmid pGETC for expression of the intact β subunit was constructed in two steps. First, pGETBC containing both the β and β' subunit genes (rpoBC) was treated with Nhel and Mulu to remove the entire β subunit coding sequence and a short NH2-terminal proximal sequence of the β' subunit. The missing region of rpoC was prepared by PCR using pGETBC as template and two primers, 5'-AGCCGATTGTGCTAGTCCCGGCTGAGTGTGTC-3' and 5'-GCTTGTGAAACCACATTAGG-3', and ligated, after treatment with Nhel and Mulu, with the pGETBC fragment carrying the sequence for NH2-terminal truncated rpoC. The resulting plasmid pGETC(+)N produced the β' subunit with an additional peptide of MASSDGSKS sequence at the NH2 terminus because of the presence of ATG codon on the vector (as a part of the Nhel site) near the junction with the rpoC insert (note that the original initiation codon for the β' subunit is GTG (24)).

To remove this NH2-terminal extra sequence, an additional Nhel site was introduced at the initiation codon of rpoC by oligonucleotide-directed mutagenesis of pGETC(+N). The resulting plasmid was digested with Nhel to isolate three fragments, two of which are ligated to prepare plasmid pGETC encoding the intact β' subunit without the extra sequence.

For construction of expression plasmids for various β' fragments, we first constructed a modified expression vector pGET1 from pGET by removing one of two Nhel sites, located upstream of the fl origin. The intact rpoC gene was inserted into this plasmid between Xbal and HindIII to construct pGET1C (Fig. 1).

Expression and Purification of Subunits and Subunit Fragments—RNA polymerase subunits and their fragments were all expressed in E. coli using the respective expression plasmids. The following plasmids were used: pGEMAX185 for a (5), pET21-α-C-His8 for α-C-His8, pGETB for β' (17), pGETC for β, pGEMD for σ70, and pGEMDCH for σ70-His8 (see above). For expression of wild-type β and β' fragments, plasmids were transformed into E. coli strain JM109(DE3). Overexpression and purification of α, β, β', and σ70 subunits and nontagged β' fragments were performed according to Fujita and Ishihama (25).

Preparation of the His8-tagged α subunit was carried out according to Kimura and Ishihama (14). For preparation of the His8-tagged σ70

1 The abbreviations used are: PCR, polymerase chain reaction; NTA, nitrotri-acetic acid; PAGE, polyacrylamide gel electrophoresis.
subunit and His₆-tagged β fragments, a batch procedure of Ni²⁺-NTA affinity purification was employed. In the case of His₆-tagged β fragments, the proteins in precipitate fractions of expressed cell lysates were solubilized with a dissociation buffer (50 mM Tris-HCl, pH 8.0, at 4 °C, 0.2 mM KCl, 10 mM MgCl₂, 1 mM EDTA, 10 mM dithiothreitol, 5% (v/v) glycerol, and 6 M urea) (25). After centrifugation, the supernatant was applied onto Ni²⁺-NTA agarose (Qiagen) previously equilibrated in a cleavage buffer (40 mM Tris-HCl, pH 7.8, 40 mM KCl, and 5% glycerol) at 20 °C for 5 min and then subjected to trypsin digestion with a final concentration of 5 mM.  

**TABLE I**  
**Construction of expression plasmids for β fragments**

| Plasmid constructed | Vector used | Restriction sites | Primers used (restriction sites in parentheses) | Fragments inserted |
|---------------------|-------------|-------------------|-------------------------------------------------|-------------------|
| pGETC(1–842) | pGETIC | KpnI-BamHI | C502/C304 | CFB03 |
| pGETC(1–345) | pGETIC | NruI-BamHI | C502/C303 | CF05 |
| pGETC(1–150) | pGETIC | NdeI-BamHI | C502/C308 | CF06 |
| pGETC(151–345) | pGETIC | NdeI | C503/C307 | CF08 |
| pGETC(201–515) | pGETC(346–515) | NdeI-EcoRV | C508/C306 | CF10 |
| pGETC(201–345) | pGETIC | NdeI-BamHI | C508/C309 | CF12 |
| pGETC(346–515) | pGETIC | NdeI-AgeI | C507/C301 | CF21 |
| pGETC(346–515) | pGETIC | NdeI-BamHI | C503/C306 | CF16 |
| pGETC(515–842) | pGETC(1–842) | NdeI-SphI | C504/C304 | CF17 |
| pGETC(842–1407) | pGETIC | MluI-BamHI | C501/C302 | CF18 |
| pGETC(842–1407) | pGETIC | MluI-BamHI | C501/C305 | CF01 |
| pGETC(1141–1407) | pGETIC | NdeI-Bst1107I | C505/C301 | CF19 |
| pGETC(1141–1407) | pGETIC | SacII-BamHI | C505/C301 | CF20 |
| pGETC(1–842)-CH6 | pGETIC | KpnI-BamHI | C503/C310 | CF15 |
| pGETC(1–345)-CH6 | pGETIC | NruI-BamHI | C506/C312 | CF13 |
| pGETC(1–150)-CH6 | pGETIC | NdeI-BamHI | C502/C311 | CF07 |
| pGETC(151–345)-CH6 | pGETIC | NdeI-BamHI | C507/C313 | CF09 |
| pGETC(201–515)-CH6 | pGETC(346–515) | NdeI-EcoRV | C506/C309 | CF11 |
| pGETC(201–345)-CH6 | pGETIC | NdeI-BamHI | C506/C313 | CF14 |
| pGETC(346–515) | pGETIC | NdeI-BamHI | C503/C310 | CF15 |
| pGETC(515–842) | pGETC(1–842) | NdeI-BamHI | C501/C310 | CF15 |
| pGETC(842–1407)-CH6 | pGETC(1–842) | NdeI-BamHI | C501/C307 | CF02 |
| pGETC(842–1407)-CH6 | pGETC(346–1407) | NdeI-Bst1107I | C505/C301 | CF19 |

*The vectors used were treated with the indicated restriction enzymes; the DNA fragments for expression of β fragments were carried out using His₆-tagged α subunit, 0.65 nmol of the intact β subunit, and various combinations of two β subunit fragments, one at a fixed concentration (0.70 nmol) and the other at various concentrations. The reconstructed core complexes were purified by Ni²⁺-NTA affinity chromatography and subjected to SDS-polyacrylamide gel electrophoresis (PAGE) on 10% uniform gels. Proteins were stained with Coomassie Brilliant Blue R-250, and the band intensity was measured with an LAS-1000 image analyzer (Fuji Photo Film Co., Japan).**

**Competitive Reconstitution of the Core Enzyme**—Reconstitution of the core enzyme was carried out using 2.01 nmol of the His₆-tagged α subunit and 0.914 nmol each of the intact β and β' subunits in the presence of various amounts of different β' fragments. The reconstituted core complexes were purified and analyzed as above. Ni²⁺-NTA agarose binding assay—Batch mode Ni²⁺-NTA affinity chromatography was carried out as described by Tang et al. (27). The His₆-tagged β' subunit or His₆-tagged β fragments were used for isolation of both core complexes (αββ' or αββ'(fragment)) and β'β' complexes (β'αβ or β'(fragment)βα). In brief, 1.08 nmol of isolated His₆-tagged β' subunit or His₆-tagged β fragments in 1 ml of the standard dissociation buffer (25) containing 1 mM dithiothreitol was mixed with 160 μl of Ni²⁺-NTA agarose equilibrated with the same dissociation buffer. After a 2-h incubation at 4 °C, agarose beads were washed six times with 1 ml each of the reconstitution buffer to remove urea. The agarose-bound β' subunit or β fragments were mixed with 1.80 nmol of the αβ subcomplex or 2.16 nmol of αβ' subunits in 1 ml of the reconstitution buffer. After a 1-h incubation at 4 °C, the agarose beads were washed once with 1 ml of the reconstitution buffer, six times with 1 ml each of buffer D plus 25 mM imidazole, and then the proteins were eluted with 160 μl of buffer D plus 400 mM imidazole. Each fraction was analyzed by SDS-PAGE on 5–20% gradient gels. Bio-Rad Ready Gels J or on 7.5, 10, and 12.5% uniform gels.

The core complex formation was also analyzed using the His₆-tagged α subunit. In this case, 2.60 nmol of His₆-tagged α subunit, 1.18 nmol of β, and 1.18 nmol of untagged β' subunit or β fragments were mixed together in the dissociation buffer and then subjected to the reconstitution with the His₆-tagged subunit mixture, 160 μl of Ni²⁺-NTA agarose previously equilibrated in the reconstitution buffer was added and incubated for 1 h at 4 °C. The agarose beads were washed with 1 ml of the reconstitution buffer and then three times with buffer D plus 5 mM imidazole, and finally the Ni²⁺-NTA agarose-bound proteins were eluted with 160 μl of buffer D plus 400 mM imidazole.
NH₂-terminal amino acid sequencing according to Negishi polyvinylidene difluoride membranes were cut out and subjected to with Coomassie Brilliant Blue R-250. Stained protein bands on the fragments thus isolated after a 30-min treatment with trypsin were incubated with trypsin at a trypsin:substrate ratio of 1:500 (w/w), as carried out for other tryptic digests treated with trypsin for various age sites by trypsin. Limited digestion of the isolated NH₂ terminus was carried out at various trypsin concentrations in the gels were blotted onto polyvinylidene difluoride membranes, and each visible band was subjected to analysis of the regions F and G (Fig. 2B). Three fragments, bpm50 and bpm47, carried the NH₂-terminal sequence starting from residues 346 and 353, respectively, indicating that they contained the sequences corresponding to the central part of the NH₂-terminal sequence, indicating that these came from the initial NH₂-terminal fragments (for the location along the NH₂-terminal sequence, shown in Fig. 2B). Two fragments, bpm50 and bpm47, were found to contain more than two different fragments of similar sizes (Fig. 2A). Three fragments, bpm42, bpm41, and bpm39, were found to retain the original NH₂-terminal sequence, indicating that these cleavage fragments were determined from the resulting sequence: the COOH-terminal end was estimated from the fragment size and the location of potential cleavage sites by trypsin. The major products are shown by shaded bars. The same analysis was carried out for other tryptic digests treated with trypsin for various times.

RESULTS

Limited Proteolysis of the Isolated β′ Subunit

The β′ subunit of E. coli RNA polymerase consists of 1407 amino acid residues and contains a total of 186 potential cleavage sites by trypsin. Limited digestion of the isolated β′ (+N) subunit with a short extra sequence of MASSDSKS at its NH₂ terminus was carried out at various trypsin concentrations and for various times. Fig. 2A shows the time-dependent cleavage pattern at a trypsin:substrate ratio of 1:500 (w/w), as analyzed by SDS-PAGE. Essentially the same cleavage patterns were obtained for the intact β′ subunit without the extra NH₂-terminal sequence (data not shown). The tryptic fragments in the gels were blotted onto polyvinylidene difluoride membranes, and each visible band was subjected to analysis of its NH₂-terminal amino acid sequence (Fig. 2B). The sites of tryptic cleavage were determined for all major cleavage products, and the COOH termini of these fragments were estimated from the fragment sizes estimated from the migration distance on SDS-PAGE and the location of potential cleavage sites along β′ by trypsin.

During the initial stage of trypsin digestion for less than 30 min, three NH₂-terminal fragments, bpm105, bpm90, and bpm88 (NH₂-terminal fragments of beta-prime are designated as bpm), were the major cleavage products (Fig. 2A). In addition, five COOH-terminal fragments, bpc73, bpc70, bpc61, bpc57, and bpc55 (COOH-terminal fragments of beta-prime are designated as bpc) could be identified. Upon further incubation, however, these COOH-terminal fragments were all degraded within 90 min of the incubation, whereas the three NH₂-terminal fragments remained at least until 180 min of the trypsin treatment.

Fig. 2B shows the location of all of these cleavage fragments along the β′ polypeptide. Fragments bpm105, bpm90, and bpm88 consist of the NH₂-terminal proximal two-thirds of β′, and include the conserved regions A to G or F. Fragments bpc73, bpc70, bpc61, bpc57, and bpc55 consist of the COOH-terminal one-third of β′ and include the conserved regions G and H and a dispensable region between region G and H. From the location of the major tryptic fragments, shown in Fig. 2B, it was predicted that the initial trypsin cleavage took place at arginine positions 798, 799, 838, 842, 905, 933, and 943 between the regions F and G (Fig. 2B).

After prolonged incubation in the presence of trypsin, the high molecular mass cleavage products disappeared, and concomitantly a number of low molecular mass fragments smaller than 50 kDa were identified, which might be generated after secondary cleavage of the primary products. All of the visible bands, as indicated in Fig. 2A, were subjected to NH₂-terminal sequencing, and the results indicated that some bands contained more than two different fragments of similar sizes (Fig. 2A). Three fragments, bpm42, bpm41, and bpm39, were found to contain the sequences corresponding to the central part of the β′ subunit (fragments derived from the middle part of the β'-prime subunit are designated as bpc) (Fig. 2B). Fragments bpm50, bpm50, bpc47, bpc42, and bpc39 all carried the NH₂-terminal sequence starting from residue Thr-934 or Ala-944.

Taking all of the sequence data together, the secondary cleavage sites could be identified as follows. (i) The initial NH₂-terminal fragments were cleaved at residue Lys-345 or Arg-352 between the conserved regions B and C, leading to generation of the NH₂-terminal proximal fragments (bpm42, bpm41, and bpm39) including the regions A to B, and the middle fragments (bpm50 and bpm47) including the regions C, D, and E. (ii) The initial COOH-terminal fragments were processed from both NH₂ and COOH termini, i.e. at residues Arg-933 or Arg-943 from NH₂ terminus and at residues approximately downstream from residue 1300 (see Fig. 2B).

Identification of β′ Fragments with Binding Activity to the αβ Complex

Use of His₆-tagged α Subunit for Complex Isolation—To identify the contact site(s) of the β′ subunit with α and β subunits, the core enzyme reconstitution experiment was carried out using a His₆-tagged αβ subunit and mixtures of tryptic-treated β′ subunit fragments. The addition of the His₆ tag at the COOH terminus of wild-type α subunit does not interfere with the assembly of core enzyme (however, some α mutants
become inactive in the assembly by the addition of His$_6$ tag (14)). Subunits were mixed in the dissociation buffer; after dialysis against the reconstitution buffer to remove urea, the subunit mixtures were subjected to Ni$^{2+}$-NTA affinity chromatography. Almost all major β' subunit fragments, including those not overlapping each other, were recovered in the complex fractions, which were eluted only in the presence of high concentrations of imidazole (data not shown). These β' subunit fragments did not bind to the Ni$^{2+}$-NTA agarose in the absence of His$_6$-tagged α and β subunits. The binding of nonoverlapping β' fragments with the αβ complex indicates the presence of multiple contact sites on the β' subunit with α and/or β subunits. However, it is not excluded yet that some β' fragments bind to either α or β subunits in a nonspecific manner.

Use of His$_6$-tagged β' Fragments for Complex Isolation—To confirm the results obtained above, we next carried out the core enzyme reconstitution experiment using α subunit, β subunit, and His$_6$-tagged β' fragments and analyzed the proteins associated with each β' fragment. For this purpose, a series of expression plasmids was constructed, which overexpressed a total of 13 species of the β' subunit fragment, each with a His$_6$ tag at the COOH terminus. All of these His$_6$-tagged β' fragments were purified to apparent homogeneity under denatured conditions in the presence of urea from inclusion bodies of the expressed cell extracts using Ni$^{2+}$-NTA agarose (data not shown). Isolated β' fragments were bound to Ni$^{2+}$-NTA agarose and, after washing with the reconstitution buffer, mixed with the αβ complex for reconstitution. The core complexes were eluted using buffer D containing imidazole and analyzed by SDS-PAGE. The elution patterns are shown in Fig. 3.

Almost all of the β' fragments, except for β'(346–515)C-His$_6$ and β'(842–1140)C-His$_6$, formed core complexes with the αβ binary complex. Because β'(346–515)C-His$_6$ and β'(842–1140)C-His$_6$ fragments did not form the core enzyme, the contact sites on the β' subunit involved in core enzyme assembly could be mapped in three regions: the NH$_2$-terminal region (residues 1–345), the middle region (residues 515–842), and the COOH-terminal region (1141–1407). Among the three candidate regions for binding of the αβ complex, the involvement of the NH$_2$-terminal region was not yet clear because the His$_6$-tagged β' fragments from the NH$_2$-terminal proximal region did not associate with Ni$^{2+}$-NTA agarose, presumably because the His$_6$ tag was not exposed on the protein surface. In addition, most of the NH$_2$-terminal fragments tended to aggregate after isolation, thereby preventing the accurate estimation of complex formation with the αβ complex (data not shown).

Use of Recombinant β' Fragments and His$_6$-tagged a Subunit for Complex Isolation—To avoid the confusion arising from the nonspecific aggregation of some β' fragments, we next added the His$_6$ tag to the COOH terminus of the α subunit and repeated the core enzyme reconstitution experiments. For this purpose, the same set of 13 different β' fragments without the His$_6$ tag were purified to apparent homogeneity (data not shown). Purified αC-His$_6$ β subunit, and either intact β' subunit or one of the β' fragments were mixed under the denatured condition at the α:ββ' molar ratio of 2.2:1.1–4. To prevent aggregation, the concentration of β' fragments was kept below 0.14 mg/ml. If there are multiple contact sites on the β' subunit, the affinity of fragments with only one contact site may be weaker than the intact β' subunit. Thus, β' fragments were added up to 4-fold molar excess over the stoichiometric molar ratio (2:1 for α:ββ'). Core complexes (or pseudo-core enzymes) were reconstituted after removing urea by dialysis against the reconstitution buffer, isolated by Ni$^{2+}$-NTA agarose chromatography, and analyzed for the subunit composition by SDS-PAGE. The elution patterns from Ni$^{2+}$-NTA agarose are shown in Fig. 4.

Among 13 different β' fragments without a His$_6$ tag tested, two fragments, β'(346–515) and β'(842–1140), did not form core complexes, in good agreement with the above results using the His$_6$-tagged β' fragments. In addition, two NH$_2$-terminal fragments, β'(1–345) and β'(151–345), did not form core complexes. However, two NH$_2$-terminal fragments, β'(1–150) and β'(201–345), formed core complexes. The results altogether support the above conclusion that two major and one minor region of the β' subunit are involved in binding of the αβ complex. However, the NH$_2$-terminal proximal minor contact region seems to be composed of two separate segments, β'(1–150) and β'(201–345), whereas the spacer region (151–200) seems to interfere with β' binding to the αβ complex.

Specificity of the β' Fragment Binding to the αβ Complex

Competition Assay of the Core Enzyme Reconstitution by the β' Fragments—As an attempt to examine the specificity of β' fragment binding to the αβ complex, we next carried out a competition assay of core enzyme reconstitution by various β' fragments constructed as above. Purified αC-His$_6$ and intact β and β' subunits were mixed in the dissociation buffer at an input molar ratio of 2.2:1:0.1 and in the presence of various amounts of one of the β' fragments. To prevent protein aggre-
Simultaneous Assembly of Two Different β’ Fragments—To confirm that two different β’ subunit fragments each including a different αβ binding domain can bind independently to the αβ complex, we next carried out mixed reconstitution experiments in the presence of two kinds of β’ fragments in various combinations. Two split fragments of the β’ subunit, β’(1–842) and β’(842–1407), were simultaneously assembled into the same core enzyme as estimated from the molar ratio of assembled β’ fragments over the α and β subunits (Fig. 5B, lane 1). Likewise, the simultaneous assembly of two different β’ fragments was observed in the following combina-
tions: β’(1–842) plus β’(1141–1407) (Fig. 5B, lane 2), β’(346–842) plus β’(842–1407) (Fig. 5B, lane 3), and β’(346–842) plus β’(1141–1407) (Fig. 5B, lane 4). Next we carried out the competition binding assay between different β’ fragments. For all of the combinations of β’ fragment mixtures noted above, the input level of the NH2-terminal proximal β’ fragment was increased up to a 4-fold molar excess over the COOH-terminal β’ fragment, keeping the NH2-terminal β’ fragment at a fixed concentration. In all of the combinations examined, the assembled level of the NH2-terminal β’ fragment stayed at a plateau even after addition of a 4-fold

Reconstitution of core complexes (αCHβ + β’)

![Image](http://www.jbc.org/Downloadedfrom)

FIG. 5. Simultaneous assembly of two different β’ fragments into the core enzyme. Panel A, competitive inhibition of the core enzyme assembly by the β’ fragments. To a standard reconstitution mixture consisting of His6-tagged α (2.01 nmol) and intact β (0.914 nmol) and β’ (0.914 nmol) subunits, 124 μg each of the indicated β’ fragments was added, which corresponded to the 1:1 weight ratio with 0.914 nmol of the intact β subunit added. The core complexes containing the His6-tagged α subunit were isolated by Ni2+-NTA affinity chromatography, and the protein composition was analyzed by SDS-PAGE (5%). The gel was stained with Coomassie Brilliant Blue R-250, and the band intensity was measured with an LAS-1000 image analyzer. The amounts of intact β’ subunit relative to that of β subunit were measured for each sample, and the levels of β’ subunit bound in the presence of the indicated β’ fragments were compared with that in the absence of β’ fragment addition. Panel B, simultaneous assembly of two different β’ fragments. Reconstitution of the core complex was carried out using 1.87 nmol of His6-tagged α subunit, 0.85 nmol of the intact β subunit, and 3.40 nmol each of two different β’ subunit fragments, β’(1–842) + β’(842–1407) (lane 1), β’(1–842) + β’(1141–1407) (lane 2), β’(346–842) + β’(842–1407) (lane 3), and β’(346–842) + β’(1141–1407) (lane 4). The reconstituted core complexes were purified by Ni2+-NTA affinity chromatography and subjected to SDS-PAGE on 10% uniform gels.
molar excess of the COOH-terminal $\beta'$ fragment (data not shown). The same type of experiment was performed by increasing the COOH-terminal $\beta'$ fragment up to 4-fold over the NH$_2$-terminal $\beta'$ fragment, which was kept at a fixed level. Again the level of assembled NH$_2$-terminal $\beta'$ fragment stayed constant irrespective of the increase of COOH-terminal fragment. Results altogether indicate that two different $\beta'$ fragments, each containing a different $\alpha\beta$ contact domain, can be assembled into the core enzyme complex simultaneously and independently.

**Identification of $\beta'$ Fragments with Binding Activity to the $\sigma^{70}$ Subunit**

**Use of His$_{6}$-tagged $\sigma^{70}$ Subunit for Complex Isolation—Isolated $\beta'$ subunit forms complexes with the $\sigma^{70}$ subunit (19). Previously we identified some $\beta'$ subunit fragments with the binding activity of the $\sigma^{70}$ subunit (20). The $\sigma^{70}$ subunit contact site on $\beta'$ was further confirmed after mapping the contact-dependent cleavage sites in the $\beta'$ subunit by $\sigma^{70}$ subunit-conjugated FeBABB (22). To confirm the previous results and to test the reliability of the contact site mapping method employed, we also carried out complex formation between tryptic cleavage fragments of the $\beta'$ subunit and the His$_{6}$-tagged $\sigma^{70}$ subunit.**

The His$_{6}$-tagged $\sigma^{70}$ subunit was purified from expressed *E. coli* cells in soluble form. For complex formation, the purified His$_{6}$-tagged $\sigma^{70}$ subunit was mixed with each of the trypsin-treated $\beta'$ subunits under nonnudnatred conditions, and the $\beta'$(fragment)$\sigma^{70}$ complexes formed were isolated using Ni$^{2+}$-NTA agarose. As in the case of core complex formation, almost all of the $\beta'$ subunit fragments were recovered in the complex fractions with the $\sigma^{70}$ subunit (data not shown). Using mixtures of $\beta'$ fragments, it was difficult to separate $\sigma^{70}$ subunit-associated $\beta'$ fragments from those bound indirectly through formation of complexes between $\beta'$ fragments. **Use of His$_{6}$-tagged $\beta'$ Fragments for Complex Isolation—To avoid this confusion, we next carried out the $\beta'$(fragment)$\sigma^{70}$ complex formation experiment using the intact $\sigma^{70}$ subunit and His$_{6}$-tagged $\beta'$ fragments and analyzed the proteins associated with each $\beta'$ fragment.** The denatured $\beta'$ fragments were first bound to Ni$^{2+}$-NTA agarose, and after renaturation by soaking with the reconstitution buffer, mixed with $\sigma^{70}$ subunit. The $\beta'$(fragment)$\sigma^{70}$ complexes were eluted with buffer D containing 400 mM imidazole.

Among 13 different species of the $\beta'$ fragment, one NH$_2$-terminal proximal fragment, $\beta'$(1–150)C-His$_{6}$, did not bind to the Ni$^{2+}$-NTA agarose, presumably because the His$_{6}$ tag was buried within the protein molecule, or this His$_{6}$-tagged $\beta'$ fragment formed aggregates. Almost all $\beta'$ fragments except for two, $\beta'$(346–515)C-His$_{6}$ and $\beta'$(842–1140)C-His$_{6}$, were found to bind the $\sigma^{70}$ subunit (Fig. 6). The basic $\beta'$ protein tends to form nonspecific complexes with the acidic $\sigma$ subunit (19), but these complexes may be dissociated upon increase in salt concentrations. To test this possibility, we then washed the Ni$^{2+}$-NTA agarose-bound $\beta'$(fragment)$\sigma^{70}$ complexes with a high salt buffer (Fig. 7). All of the fragments derived from the middle portion and the COOH-terminal region of $\beta'$ subunit were eluted by washing with the high salt elution buffer (buffer D, 25 mM imidazole plus 0.3 M KCl), indicating that the binding affinity is weak and presumably nonspecific for these fragments. On the other hand, the NH$_2$-terminal fragments, $\beta'$(1–345)C-His$_{6}$, $\beta'$(151–345)C-His$_{6}$, $\beta'$(201–515)C-His$_{6}$, and $\beta'$(201–345)C-His$_{6}$, were retained tightly onto the Ni$^{2+}$-NTA agarose even after washing with the high salt elution buffer, but they could be eluted by washing with the elution buffer containing 400 mM imidazole. Thus, we concluded that the NH$_2$-terminal portion of the $\beta'$ subunit is involved in the specific binding of the $\sigma^{70}$ subunit.

**DISCUSSION**

**Proteolytic Cleavage Map of the $\beta'$ Subunit—**The $\beta'$ subunit of *E. coli* RNA polymerase is a large basic protein consisting of 1407 amino acid residues and forms, together with the $\alpha$ and $\beta$ subunits, the core enzyme with the subunit composition $\alpha_{2}\beta\beta'$. In the assembled core enzyme, the $\beta'$ subunit plays a major role in initial nonspecific binding of the RNA polymerase to template DNA. The isolated $\beta'$ subunit is totally inactive, and its intrinsic activities are exposed, in a stepwise manner, after formation of the $\alpha_{2}\beta$ complex and the core enzyme (for review, see Ref. 2). In contrast, the isolated $\beta'$ subunit retains the binding activities of DNA and the $\sigma^{70}$ subunit (19), suggesting that the conformation of isolated $\beta'$ subunit is close to that assembled in the RNA polymerase core enzyme. In agreement with this interpretation, the initial proteolytic cleavage sites of isolated $\beta'$ subunit analyzed in this study are close to the cleavage sites of the $\beta'$ subunit in the core enzyme (28).

The initial cleavage sites are located between Arg-798 and
Arg-943 near one of the two split sites of archaeabacterial β′ homologs (29, 30). This region corresponds to the spacer region between two subunit-subunit contact domains with the σ70 subunit and the αβ complex (see below), suggesting that this region is exposed on the surface of RNA polymerase. As illustrated in Fig. 8, a number of functional sites are located within or near this trypsin-sensitive region, including the binding site for streptolydigin, a potent inhibitor of transcription elongation (31), the epitope for inhibitory monoclonal antibodies (32) and for streptolydigin, a potent inhibitor of transcription elongation (36). These observations altogether suggest that the proteolytic cleavage-sensitive region of the β′ subunit is exposed on the surface of core enzyme and is involved in the catalytic functions of RNA polymerase.

Subunit-Subunit Contact Sites on the β′ Subunit with the αβ Complex—Two separate fragments of the β′ subunit showed strong affinity to the αβ complex, one β′(515–842) and the other β′(1141–1407) (see Fig. 8). The fragment β′(515–842) derived from the central portion of β′ corresponds to the segment between the split sites of β′ homologs of chloroplasts (37–39) and those of archaeabacteria (29, 30). In agreement with the concept that the core polypeptides within multisubunit complexes such as the RNA polymerase β′ subunit have mosaic structures consisting of two functional domains, one for protein-protein contacts and the other for functions such as the catalytic activities for RNA polymerization, each being comprised of noncontiguous segments on the primary structure (17), the β′ subunit is also composed of two distinct functional domains. As noted above, the downstream sequence is involved in the catalytic functions of RNA polymerase. In addition, several lines of evidence indicate the involvement of upstream sequence in RNA synthesis. (i) The binding sites of Mg2+ (residues 457–464) (40, 41), located upstream of the central αβ complex binding site, form a part of the catalytic site of RNA polymerization. (ii) The 3′ end of nascent RNA can be cross-linked to both upstream (400–467) and downstream (932–1020) residues of this αβ-contact site (33, 34). (iii) The termination-altering mutations are located in both upstream and downstream regions of the subunit assembly domain (35). The second tight contact site of the αβ complex is located at the extreme COOH-terminal end of the β′ subunit. Again, this region is involved in protein-protein interactions with not only the αβ complex but also the σ70 subunit (see below) and single-stranded DNA-binding protein of phage N4 (42), which plays a key role in specificity modulation of host RNA polymerase in phage-infected E. coli.

In addition to these two tight contact sites on β′ with the αβ complex, the extreme NH2-terminal proximal fragment, β′(1–345), showed a weak activity of αβ binding. However, both NH2- (β′(1–150)) and COOH-terminal (β′(201–345)) subfragments derived from this region showed higher activities of the αβ binding, indicating that the internal segment between residues 151 and 201 interferes with the interaction of this NH2-terminal region with the αβ complex. One possibility is that the interaction between the NH2-terminal region of β′ and the αβ complex varies depending on the conformational change of β′ during RNA polymerase assembly or discrete steps of transcription. For instance, the major contact site of the β′ subunit with the σ70 subunit is located in the COOH-terminal proximal segment (residues 201–345), and thus, the αβ complex binding activity of the extreme NH2-terminal segment (residues 1–150) could be largely affected depending on whether the σ70 subunit is associated (holoenzyme form) or not (core enzyme form). In the NH2-terminal proximal segment, there is a putative zinc binding motif (see Fig. 8) which is believed to play a role in recognition of some rho-independent termination signals because mutations in this region affect transcription termination (43).

We also tested the reconstitution of core subunit complexes from pairwise combinations of the β′ fragments. Preliminary experiments suggested that two different β′ fragments with αβ binding activity can bind to the αβ complex simultaneously and independently (data not shown).

**Subunit-Subunit Contact Sites on the β′ Subunit with σ70 Subunit**—The β′σ70 binary complex is formed in vitro from isolated individual components (19). Mapping of the contact sites on β′ subunit with the σ70 subunit was therefore performed by measuring complex formation of β′ fragments and the intact σ70 subunit (20). Here we extended this approach using a larger collection of β′ fragments with or without the His9 tag. Luo et al. (20) reported that the region of β′ between residues 201 and 477 is required for binding of the σ70 subunit, and here we identified a smaller fragment covering residues 201–345 which is still capable of binding the σ70 subunit. We also carried out mapping of the σ70 subunit contact site by analysis of the contact-dependent cleavage sites on β′ with the
interaction between the core enzyme and the host N4 single-stranded DNA-binding protein modulate the in-
phage P2 growth (45). One possibility is that phage factors such as RNA polymerase, resulting in the loss of ability to support
minus region affects an as yet unidentified function(s) of the
binding protein (43). Likewise, a truncation of the COOH ter-
minus region downstream from residue 1141. The
binding sites with the
strong contact site, however, we identified in this study two
weak binding sites with the
strong binding sites with the
contact sites are indicated by black bars; the secondary and weak contact sites are shown by gray bars. Both the eight conserved sequences
among β' homologs of prokaryotic and eukaryotic RNA polymerases (46, 47) and the functional sites of E. coli RNA polymerase β' subunit so far
identified are shown along the primary sequence of E. coli β' subunit.

Among 13 different fragments of the β' subunit examined in
this study, β'(201–345) showed the strongest activity of
RNA polymerization and binding of nascent RNA. Thus, it appears that the protein-protein contact sites are clustered
within three narrow regions that are separated along the
primary structure of the β' subunit. The finding described here supports the concept that the β' subunit is composed of two
kinds of functional domain, one for protein-protein interactions and the other for functions related to transcription such as RNA polymerization and binding of nascent RNA.

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FIG. 8. The subunit-subunit contact surfaces on the β' subunit. The binding activities of 13 different kinds of the β' fragment with αβ complex and σ70 subunit are summarized. The levels of binding activity represent: + +, strong binding; +, weak binding; −, no binding. ND, not determined. The contact surfaces for the αβ complex and the σ70 subunit were estimated from the results of binding assays. The primary and strong contact sites are indicated by black bars; the secondary and weak contact sites are shown by gray bars. Both the eight conserved sequences among β' homologs of prokaryotic and eukaryotic RNA polymerases (46, 47) and the functional sites of E. coli RNA polymerase β' subunit so far identified are shown along the primary sequence of E. coli β' subunit.

Subunit-Subunit Contact Surfaces on RNA Polymerase β' Subunit

Zinc binding
αβ binding
σ70 binding
Tryptic cleavage sites
Spine of FeBABE cleavage
RNA 2'end cross-links
Protection from Fe-EDTA cleavage
Active site
RNA 2'end cross-links
N658 binding
Phase P2 growth defect
Binding activity
αβ ασσβ σ70

FIG. 8. The subunit-subunit contact surfaces on the β' subunit. The binding activities of 13 different kinds of the β' fragment with αβ complex and σ70 subunit are summarized. The levels of binding activity represent: + +, strong binding; +, weak binding; −, no binding. ND, not determined. The contact surfaces for the αβ complex and the σ70 subunit were estimated from the results of binding assays. The primary and strong contact sites are indicated by black bars; the secondary and weak contact sites are shown by gray bars. Both the eight conserved sequences among β' homologs of prokaryotic and eukaryotic RNA polymerases (46, 47) and the functional sites of E. coli RNA polymerase β' subunit so far identified are shown along the primary sequence of E. coli β' subunit.

Subunit-Subunit Contact Surfaces on RNA Polymerase β' Subunit

Among 13 different fragments of the β' subunit examined in
this study, β'(201–345) showed the strongest activity of
σ70 subunit binding. In addition to this NH2-terminal proximal strong contact site, however, we identified in this study two weak binding sites with the σ70 subunit, one in the middle of β'
between residues 515 and 842 and the other in the COOH-
terminal proximal region downstream from residue 1141. The
weak σ70 contact site in the central part of β' overlaps the region including residues 581, 613, and 728, which are
protected by binding of the σ70 subunit, from the cleavage by
dehydroxyl radicals generated by Fe-EDTA (44). On the other
hand, the COOH-terminal proximal weak σ70 binding site over-
laps the contact site with phase N4 single-stranded DNA-
binding protein (43). Likewise, a truncation of the COOH ter-
minus region affects an as yet unidentified function(s) of the
RNA polymerase, resulting in the loss of ability to support
phase P2 growth (45). One possibility is that phage factors such as N4 single-stranded DNA-binding protein modulate the in-
teraction between the core enzyme and the host σ70 subunit.

Interestingly, the two weak ασσ subunit binding sites correspond to the strong binding sites with the αβ complex (see above).
Subunit-Subunit Contact Surfaces on RNA Polymerase β' Subunit

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