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A System for Heterologous Expression and Isolation of *Escherichia coli* RNA Polymerase and Its Components

Yu. A. Khodak[1], O. N. Koroleva[2], and V. L. Drutsa[1]*

[1]*Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University, 119992 Moscow, Russia; fax: (495) 939-3181; E-mail: drutsa@genebee.msu.su; hod@freemail.ru*
[2]*Chemical Faculty, Lomonosov Moscow State University, 119992 Moscow, Russia; fax: (495) 939-3181; E-mail: koroleva@genebee.msu.su*

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Abstract—A set of plasmid vectors for expression of all major *Escherichia coli* RNA polymerase subunits as fusion proteins with intein- and chitin-binding domains, allowing protein purification in accordance with IMPACT technology, was constructed. It is demonstrated that the fusion subunits α, β, or β′ in conjunction with the natural subunits α, β, β′, and σ can participate in RNA polymerase assembly *in vivo*, providing affinity-based isolation of the enzyme. Functional activity of the enzyme preparations was demonstrated in the experiments on *in vitro* transcription and promoter complex formation. With the use of IMPACT technology, σ^{70} subunit can be isolated as an individual protein without admixture of RNA polymerase.

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Key words: *E. coli* RNA polymerase, subunits, affinity chromatography, IMPACT-system, transcription

One of the key cellular processes is transcription, which is catalyzed by DNA-dependent RNA polymerase (RNAP) [1]. *Escherichia coli* RNAP is one of the most studied enzymes of this class, with the minimal (core) enzyme consisting of five subunits: β (150.6 kD), β′ (155.2 kD), two α (36.5 kD), and ω (10.5 kD). The presence of ω subunits is not a necessary condition for functional activity of RNAP *in vitro* [2, 3]. The core enzyme is capable of transcription elongation and termination; however, the participation of an additional protein factor, σ subunit (predominantly σ^{70}, 70.2 kD), is necessary for specific initiation of transcription. Despite the intensive studies, the molecular mechanisms of transcription are not entirely clear (see, for instance, [4]). A highly informative approach to study of protein structure and function is site-directed mutagenesis. However, obtaining the mutant forms of such complex multi-subunit enzymes as *E. coli* RNAP is associated with a number of difficulties. The main difficulty is to obtain a preparation free of admixtures of native forms, thus making the development of novel methods of protein purification an important problem both for the enzyme and its subunits.

At present several approaches to RNAP isolation are known. The native enzyme is obtained by a multi-step chromatographic purification [5-8]. The methods for obtaining mutant *E. coli* RNAP include the isolation of individual recombinant subunits from overproducing strains followed by *in vitro* reconstruction of core or holoenzyme [3, 9-13]. The drawback of this approach is the possibility that a protein with native structure can be incorporated into mutant preparations. Moreover, due to propensity of large β and β′ subunits to aggregation, the major part of heterologously expressed recombinant proteins relocates from cytoplasm into inclusion bodies. Extraction of these subunits requires denaturation and renaturation steps, which results in significant loss of material, and does not guarantee the correct folding of all of the protein molecules. Different methods based on affinity chromatography have been developed for selective isolation of both individual recombinant RNAP subunits and the whole enzyme. The approach is based on incorporation of additional peptide fragments (modules) into subunits, providing specific binding to ligand immobilized on a solid support [14]. The most frequently used

Abbreviations: BSA) bovine serum albumin; CBD) chitin-binding domain; IMPACT) intein mediated purification with an affinity chitin-binding Tag; IPTG) isopropyl-β-D-thiogalactoside; KF DNAP) Klenow fragment of *E. coli* DNA polymerase I; PCR) polymerase chain reaction; RNAP) *E. coli* RNA polymerase.

* To whom correspondence should be addressed.

module is hexahistidine tag, forming chelate complexes with Ni-NTA agarose [15]. This approach was used for isolation of individual RNAP subunits, whole reconstructed enzyme, as well as its mutant forms [16-18]. A significant drawback of the preparations obtained in this way is the presence of auxiliary amino acid sequence in the protein molecule. More promising from this point of view is the IMPACT (intein mediated purification with an affinity chitin-binding Tag) system (NEB, USA), where the target protein is expressed as a fusion polypeptide, comprising two technological modules, intein and chitin-binding domain (CBD), and being able to form a strong complex with chitin fragments immobilized on agarose (Fig. 1) [19]. In this connection, the target protein either has a structure that completely corresponds to the native form or contains a minimal addition, a glycine residue. Isolation of recombinant protein in the IMPACT system occurs under conditions of preservation of its native structure and includes two highly specific stages: affinity adsorption on chitin-containing support, and mild cleavage from the adsorbed polypeptide domain, providing high purity of the resulting preparation. It should be noted, however, that under such mild conditions it is difficult to remove impurities forming relatively strong complexes with the target protein, as well as the degradation (proteolysis) products of the protein itself.

The IMPACT system has been widely used for the isolation of high purity recombinant proteins; however, it was almost not used for isolation of multi-subunit proteins and protein-containing complexes. To date only one such study has been published, where *E. coli* RNAP was isolated using β′ subunit with cleavable intein–CBD module [20].

The present work attempts to study in more detail the possibility of application of the IMPACT system for obtaining the preparation of both individual recombinant subunits and *E. coli* RNAP itself. For this purpose, a set of plasmid vectors has been constructed for expression of each of the subunits with intein–CBD module, and different variants of expression and isolation of RNAP and its components have been studied.

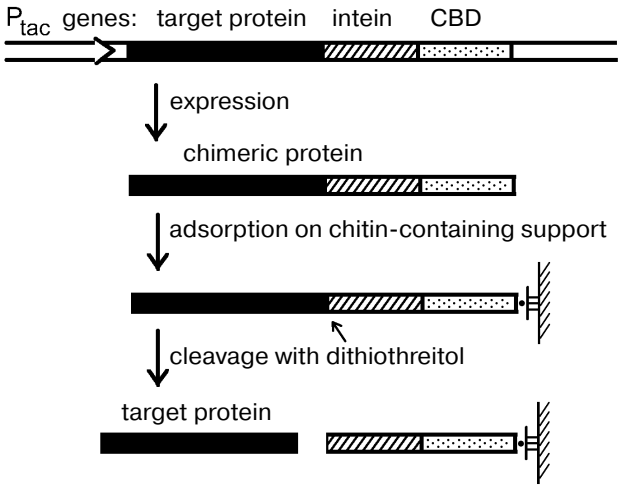

Fig. 1. Scheme for protein production using the IMPACT technology.

MATERIALS AND METHODS

In this work we used Tris and EDTA (Merck, Germany), isopropyl-β-D-thiogalactoside (IPTG) (MP Biomedicals Inc., Germany), ampicillin and dithiothreitol (DTT) (Serva, USA), acrylamide and N,N′-methylene-*bis*-acrylamide (Fluka, Switzerland), [γ-^{32}P]ATP (185 PBq/mol) and [α-^{32}P]UTP (148 PBq/mol) (Isotope, Russia), ribonucleoside triphosphates ATP, GTP, CTP, and UTP (Boehringer, Germany), bovine serum albumin (BSA), dATP, dGTP, dCTP, and dTTP (SibEnzyme, Russia), tryptone, agar, and yeast extract (Difco, USA), agarose and heparin-agarose (Sigma, USA), and protein markers and chitin-containing support (NEB).

The following enzyme preparations were used: T4 polynucleotide kinase and DNA ligase (Fermentas, Lithuania), *Thermus aquaticus* DNA polymerase (*Taq*SE polymerase) and Klenow fragment of *E. coli* DNA polymerase I (KF DNAP) (SibEnzyme), *E. coli* RNAP holoenzyme (1.1 μg/μl, 1.2 activity units/μl) (Sigma), and *E. coli* RNAP core enzyme (0.54 μg/μl, 1 activity unit/μl) (Epicenter Technologies). Restriction endonucleases were supplied by Fermentas, Gibco BRL (USA), and SibEnzyme. Enzymatic treatment of DNA was performed according to the enzyme suppliers' instructions.

Plasmids pUC18 (Fermentas) and pCYB4 (NEB) and *E. coli* strain ER1821 (NEB) were used.

The structures of all synthetic oligodeoxyribonucleotides (Sintol, Russia) used in this work are given in the table.

The main buffers used were: transcription buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 0.05 mg/ml BSA), lysis buffer (20 mM sodium phosphate, pH 7.5, 500 mM NaCl, 1 mM EDTA); buffer A (50 mM Tris-HCl, pH 7.9, 10 mM $MgCl_2$, 0.01 mM $ZnCl_2$, 1 mM EDTA); TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA).

Polymerase chain reaction (PCR) was performed in a CycloTemp-107 programmable thermostat (Resurs-Pribor Ltd., Obninsk, Russia). Optimal PCR conditions were selected empirically for each experiment. The minimal number of cycles resulting in the accumulation of sufficient amount of target product for subsequent cloning was used in each case.

DNA sequences were determined by the Sanger method on an ABI PRISM 310 automatic sequencer (Applied Biosystems, USA) using standard primers with hybridization site near the poly-linker region of pCU18

Synthetic oligodeoxyribonucleotides used in this work

Name	Primary structure (5′→3′)
p20-rpal	pCAGGGTTCTGTGACAGAGTT
p19r-rpar	pCTCGTCAGCGATGCTTGCC
27-rpbl	GCACCATGGTTTACTCCTATACCGAGA
p16r-blfr	pGGGTATCCAGATCGCC
17-bmfl	GTTCTTCTCCGAAGACC
15r-bmfr	CGGGACACACACGCC
p17-brfl	pAGTACGTGGATGCTTCC
p20r-rpbr	pCTCGTCTTCCAGTTCGATGT
p24-rpcl	pGTGAAAGATTTATTAAAGTTTCTG
p17r-clfr	pCTTTGATCATGTCGGCC
16-cmfl	GGTGGTCGTTTCGCGA
17r-cmfr	CGATTGCTTCACCCTTG
p17-crfl	pGGTCGTGTAACTGCTGA
p19r-rpcr	pCTCGTTATCAGAACCGCCC
p22-rpdl	pATGGAGCAAAACCCGCAGTCAC
p19r-rpdr	pATCGTCCAGGAAGCTACGC
25-kupl	AACCTATAAAAATAGGCGTATCACG
23-kdor	CTCTCATAATTCGCTCCATTAAG
66_1	CGTCTTCAAGAATTCCCTTGACACTTACTCATCCGGATCCTATAATGGGGGATCCGTCGACCTGCA
41_2	AGGATCCGGATGAGTAAGTGTCAAGGGAATTCTTGAAGACG
15-sqbl	ACTCGGCCCAGGCGG
15-sqcl	CCGTGCACCGACTCT
15r-sqcr	TCGCGGTGATTGGCG
16-sqdl	GAGCTTTCCCAGGAAG

and pCYB4, primers for PCR amplification of gene fragments of RNAP subunits, and primers 15-sqbl, 15-sqcl, 15r-sqcr, and 16-sqdl.

Preparative 5′-phosphorylation of oligodeoxynucleotides using T4 polynucleotide kinase and ATP, as well as incorporation of 5′-^{32}P-label in the presence of [γ-^{32}P]ATP was carried out following a published protocol [21].

A DNA fragment of 180 bp containing two divergent consensus promoters was obtained by PCR using pKD-2 plasmid (a derivative of pAA224 [22]) as a template and primers 25-kupl and 23-kdor.

Labeled DNA duplex 5′-[^{32}P]66_1:41_2 was obtained by heating the mixture of oligonucleotides 5′-[^{32}P]p66_1 and 41_2 at the molar ratio 1 : 1.2 for 10 min at 75°C in transcription buffer without BSA followed by slow (1.5 h) cooling to 20°C.

Electrophoresis of polynucleotides was performed in 8% denaturing polyacrylamide gel using glass plates (25 × 20 × 0.04 cm) in Tris-borate buffer (pH 8.3) containing 8 M urea, at electric field intensity of 50 V/cm.

Electrophoresis of DNA–protein complexes was performed in 4% non-denaturing polyacrylamide gel using glass plates (25 × 20 × 0.08 cm) in Tris-borate buffer (pH 8.3) without urea, under electric field intensity of 20 V/cm.

Electrophoretic analysis of protein preparations was performed by the Laemmli method [23] in 8% denaturing SDS-polyacrylamide gel. The gels were stained with zinc-imidazole as described in [24]. The gel was rinsed with distilled water (three times for 5 min), soaked in 0.2 M aqueous imidazole solution (5-10 min), incubated in 0.3 M aqueous $ZnCl_2$ solution until color development (1-2 min), rinsed with water, and photographed against a dark background. Radioautographs were obtained on a Phosphor Imager (Molecular Dynamics, USA).

Protein preparations were concentrated in Ultrafree-MC ultrafiltration centrifuge wells (Millipore, USA). In the case of α subunit preparations wells with molecular weight cut off 10 kD were used, in the case of σ subunit – 30 kD, and β and β' subunits and RNAP – 100 kD.

Escherichia coli cells were cultivated in dyt medium (16 g tryptone, 10 g yeast extract, 5 g NaCl per liter); transformation, cloning, isolation of genomic and plasmid DNA, purification of DNA fragments in agarose gel, as well as other gene engineering manipulations were carried out using standard methods [25].

Amplification of genes coding for *E. coli* RNAP subunits. *Escherichia coli* genomic DNA (0.5 μg/μl) was used as a template for PCR. All DNA fragments for subsequent cloning were purified by electrophoresis in agarose gel.

PCR product of α subunit gene without ATG- and stop codons with a length of 984 bp (fa) was obtained using primers 20-rpal and 19r-rpar (12 cycles: 94°C, 30 sec; 62°C, 30 sec; 72°C, 60 sec).

PCR product of σ subunit gene without stop codons with a length of 1839 bp (fd) was obtained using primers 22-rpdl and 19r-rpdr (13 cycles: 94°C, 30 sec; 60°C, 30 sec; 72°C, 120 sec). The $fd2_N$-fragment (321 bp) was obtained by the cleavage of *fd* PCR product with *Nco*I restriction endonuclease.

PCR product of β subunit gene was obtained as three overlapping fragments: fb1 (1464 bp; primers 27-rpbl and 16r-blfr; 12 cycles: 94°C, 30 sec; 52°C, 30 sec; 72°C, 120 sec), fb2 (1359 bp; primers 17-bmfl and 15r-bmfr; 11 cycles: 94°C, 30 sec; 54°C, 30 sec; 72°C, 120 sec), and fb3 (1853 bp; primers 17-brfl and 20r-rpbr; 12 cycles: 94°C, 30 sec; 54°C, 30 sec; 72°C, 120 sec). *Prior to* the isolation of fb1 PCR fragment, a short auxiliary sequence was removed by *Nco*I restriction endonuclease, resulting in fragment $fb1_N$.

PCR product of β′ subunit gene was also obtained as three fragments: fc1 (997 bp; primers 24-rpcl and 17r-clfr; 11 cycles: 94°C, 30 sec; 54°C, 30 sec; 72°C, 120 sec), fc2 (1978 bp; primers 16-cmfl and 17r-cmfr; 11 cycles: 94°C, 30 sec; 54°C, 30 sec; 72°C, 120 sec), and fc3 (1701 bp; primers 17-crfl and 19r-rpcr; 12 cycles: 94°C, 30 sec; 54°C, 30 sec; 72°C, 120 sec).

Construction of auxiliary plasmids and subcloning. The PCR fragments fb2, fc2, and fd were subcloned into *Sma*I-digested pUC18 plasmid. After confirmation of insert structures by sequencing the auxiliary plasmids in the case of fb2-fragment were treated with *Cla*I and *Rsr*II restriction endonucleases, resulting in generation of $fb2_{CR}$-fragment (1058 bp); in the case of fc2 – with *Sna*BI and *Sal*I, resulting in generation of $fc2_{SS}$ (1752 bp); in the case of fd – with *Nco*I resulting in generation of $fd1_{NN}$ (1518 bp).

Construction of expression plasmids. The general scheme for the construction of expression plasmids based on pCYB4 is shown in Fig. 2.

To assemble the expression plasmid pC4-a, carrying the gene of α subunit, the pCYB4 plasmid was cleaved with *Nco*I and *Sma*I restriction endonucleases, filled with KF DNAP, and linked with the fa-fragment using DNA ligase. The search for clones with correct orientation of the insert was performed by PCR. The structure of the insert region was confirmed by sequencing.

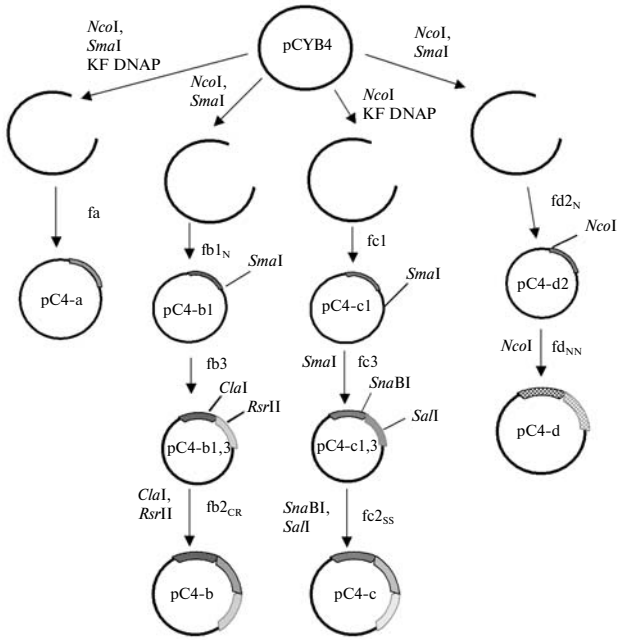

Fig. 2. Scheme for construction of pC4-a, pC4-b, pC4-c, and pC4-d expression plasmids containing the genes coding for α, β, β′, and σ subunits of *E. coli* RNAP. The enzymes used for DNA treatment and the consecutive steps of insertion of genes or gene fragments are shown.

To construct the expression plasmid pC4-d containing the gene of σ subunit, the pCYB4 plasmid was digested with *Nco*I and *Sma*I restriction endonucleases and ligated with $fd2_N$-fragment. The structure of the insert region was confirmed by sequencing. Intermediate pC4-d2 plasmid was cleaved by *Nco*I restriction endonuclease and coupled to $fd1_{NN}$-fragment. Clones with correct orientation of the insert were selected by PCR.

To obtain pC4-b expression plasmid with β subunit gene, the pCYB4 was cleaved with *Nco*I and *Sma*I restriction endonucleases, filled in with KF DNAP, and coupled to fb1-fragment. The structure of the insert was confirmed by sequencing. Intermediate pC4-b1 was digested with *Sma*I and coupled to fb3-fragment. Clones with correct orientation of the insert were selected by PCR. The structure of the insert was confirmed by sequencing. The intermediate pC4-b1,3 plasmid was digested with *Cla*I and *Rsr*II restriction endonucleases and ligated with $fb2_{CR}$-fragment.

To construct pC4-c expression plasmid containing the gene of β′ subunit, the pCYB4 plasmid was cleaved with *Nco*I restriction endonuclease, filled in with KF DNAP, and ligated with fc1. Clones with correct orientation of the insert were selected by PCR. The structure of the insert was confirmed by sequencing. Intermediate pC4-c1 plasmid was cleaved with *Sma*I and coupled to

fc3-fragment. Clones with correct orientation of the insert were selected by PCR. The structure of the insert was confirmed by sequencing. The intermediate pC4-c1,3 plasmid was cleaved with *Sna*BI and *Sal*I restriction endonucleases and ligated with $fc2_{SS}$-fragment.

Isolation of soluble proteins. To 50 ml of dyt-medium containing ampicillin (100 μg/ml), 2 ml of overnight *E. coli* ER1821 cell culture with corresponding plasmids was added, and the culture was grown in roller bottles for 1-1.5 h at 35°C until A_{600} reached 0.6-0.8. Then IPTG (2 mM) was added, and the cells were grown for an additional 2 h. The cells were pelleted by centrifugation, supernatant was removed, the pellet resuspended in 0.8 ml of lysis buffer, and the cells were disrupted by sonication. The resulting cell homogenate was centrifuged (15 min, 4°C, 12,500*g*). Cleared homogenate was applied to a column with 0.1 ml of chitin-containing support. The column was washed with lysis buffer (3 times with 0.2 ml), buffer A (3 times with 0.2 ml), and buffer A (0.1 ml, rapidly) with 50 mM DTT. The same buffer (0.05 ml) was added on the top of the column and allowed to proceed for 16-20 h at 4°C. Detached proteins were eluted from the column with buffer A (3 times with 0.1 ml). The resulting protein preparations were subsequently analyzed.

All protein preparations were concentrated by ultrafiltration, transferred into buffer A, and stored at −20°C in buffer A with glycerol (50%).

Chromatography on heparin-agarose. Protein preparation obtained after elution from chitin-containing resin was applied to a column with 0.25 ml of heparin-agarose. The column was washed with 0.2 M NaCl in TE buffer (6 times with 0.75 ml), and RNAP was eluted with 0.6 M NaCl in TE buffer (3 times with 0.25 ml). The resulting preparation was concentrated by ultrafiltration (using a membrane with molecular weight cut off 100 kD) followed by transfer into buffer A and electrophoretic analysis in 8% SDS-polyacrylamide gel.

Isolation of proteins from cell debris. Cell debris pellet obtained after centrifugation of cell lysates from 2 ml of IPTG-induced culture was resuspended in 0.5 ml of lysis buffer and centrifuged (10 min, 4°C, 12,500*g*) followed by removal of supernatant. The procedure was repeated five times. The washed pellet was resuspended in 0.2 ml of 8 M aqueous urea. The suspension was centrifuged (15 min, 4°C, 12,500*g*), and the supernatant was analyzed by SDS-PAGE in 8% gel.

Saturation of RNAP with σ subunit. RNAP preparations (50-100 nM) were incubated in transcriptional buffer with 6-fold molar excess of σ subunit for 30 min at 30°C. Holoenzyme from commercial preparation of RNAP core enzyme was reconstructed in the same way.

Study of formation of RNAP complexes with DNA. Labeled DNA duplex 5′-[^{32}P]$p66_1$:41_2 (1-10 nM) was incubated with the protein preparation under examination (10-100 nM) in 10 μl of transcriptional buffer for 15 min at 37°C. Then 1 μl of 50% glycerol with marker dyes xylene cyanole (0.01%) and bromophenol blue (0.01%) was added, the mixture was loaded in the wells of 4% non-denaturing polyacrylamide gel, and electrophoresis was performed while avoiding gel heating. The gel was dried and radioautographed.

In the experiments revealing the formation of specific RNAP–promoter complexes, the reaction also contained heparin at final concentration 100 μg/ml added at the same time as glycerol.

***In vitro* transcription.** Protein preparations containing 50-100 nM RNAP were incubated with 180 bp DNA template (5-10 nM) for 10 min at 37°C in 6 μl of transcription buffer. Then 2 μl of the same buffer containing the mixture of labeled ribonucleoside triphosphates (final concentrations: 0.1 mM ATP, GTP, and CTP; 0.05 mM UTP; 10 kBq [α-^{32}P]UTP) was added. In the case of single-round transcription, heparin (final concentration 100 μg/ml) was also added. The mixture was incubated for 10 min at 37°C, and transcription reaction was terminated by addition of 6 μl of stop solution (0.01% xylene cyanole and 0.01% bromophenol blue, 25 mM EDTA in 80% aqueous formamide). The mixture was loaded on 8% denaturing polyacrylamide gel and analyzed by electrophoresis, and the gel was dried and radioautographed.

RESULTS AND DISCUSSION

To assemble target construct the genes coding for RNAP subunits were amplified by PCR directly from genomic DNA using the primers designed based on a nucleotide sequence of *E. coli* genome available at GenBank (http\\:www.ncbi.nlm.nih.gov, number NC_000913). To obtain a PCR amplification product of *E. coli* nucleotide sequence coding for RNAP subunits, special approaches have been used to avoid undesirable nucleotide substitutions (mutations), which might have emerged during the amplification. Thus, the genes of the large subunits were obtained as overlapping PCR fragments. In all cases such PCR conditions were selected that would require the minimal number of cycles necessary for obtaining sufficient amount of product for subsequent cloning. Moreover, DNA polymerase with proofreading 3′→5′ nuclease activity was used.

Plasmids for expression of *E. coli* RNAP subunits containing the "technical module", intein–CBD domain, were constructed based on pCYB4 vector, where the transcription of the target gene is under the control of the IPTG-inducible *tac*-promoter [19]. The scheme for construction is shown in Fig. 2. The *rpoA* gene encoding the α subunit was obtained as a single PCR fragment and cloned in pCYB4 vector, resulting in formation of expression plasmid pC4-a. The *rpoD* gene encoding the σ subunit was obtained as one large PCR fragment and subcloned in pUC18 plasmid, since it was not possible to

insert the entire fragment in pCYB4 vector. The construction of expression plasmid pC4-d was performed in two steps. First, 3′-terminal region of the PCR fragment was inserted into pCYB4 vector digested with *Nco*I and *Sma*I restriction endonucleases; then 5′-terminal region of the PCR fragment was incorporated into the resulting intermediate plasmid cleaved with *Nco*I. The strategy for avoiding the complications during amplification of such large genes as *rpoB* (4026 bp) and *rpoC* (4221 bp) was chosen for construction of expression plasmids pC4-b and pC4-c. PCR amplification products for these genes were obtained as three overlapping fragments. The construction of expression plasmids pC4-b and pC4-c was performed in three steps. First, 5′-terminal region of PCR fragment was incorporated into pCYB4 vector, after that 3′-terminal region of PCR fragment was inserted into intermediate plasmid, and then the central region of PCR fragment was introduced in the second intermediate plasmid. The identity of plasmid sequence in the regions coding for *E. coli* RNAP subunits to that of the corresponding native genes was confirmed by sequencing.

Expression and isolation of target proteins was performed using *E. coli* ER 1821 strain and the standard protocol for the IMPACT system: after cell growth, induction, collection, and disruption, the cleared homogenate containing all soluble cellular proteins was applied to a column with chitin-containing resin; the resin was then thoroughly washed and bound proteins were detached from the intein–CBD domain, strongly adsorbed on the resin, with DTT. Purity of the resulting protein preparations was analyzed by SDS-PAGE (Fig. 3). The nucleotide sequences of *E. coli* RNAP subunits isolated using this technology are completely identical to their natural analogs, with the exception of an additional glycine residue at the C-terminus, which should not have a significant effect on their properties.

It was supposed that the α and σ subunits linked with intein–CBD domain can be accumulated in cell cytoplasm in large quantities during heterologous expression. Indeed, significant amounts of both α and σ subunits were successfully isolated from cells with pC4-a and pC4-d plasmids using the IMPACT technology applied to cleared homogenate after centrifugation. It should be mentioned that the σ subunit was isolated as a rather pure individual protein (Fig. 3, lane *6*). In the case of the α subunit, electrophoretic analysis of proteins eluted from chitin-containing column after cleavage of intein–CBD domain demonstrated the presence (in addition to the main protein product, α subunit) of a quantity of β, β′, and σ subunits (the presence of ω subunit was not checked) (Fig. 3, lane *3*).

Contrary to the small subunits of *E. coli* RNAP, prone to aggregation large β and β′ subunits, when coupled with rather big intein–CBD domain, could be almost completely deposited in inclusion bodies, as earlier observed for heterologous expression of free β and β′ subunits [3, 11]. Indeed, the pellets obtained after centrifugation of homogenates of cells containing plasmids pC4-b and pC4-c and solubilized in 8 M urea solution contained large amounts of proteins with molecular weights of ~205 and 210 kD (estimated by electrophoretic mobility), which correspond to molecular weights of β

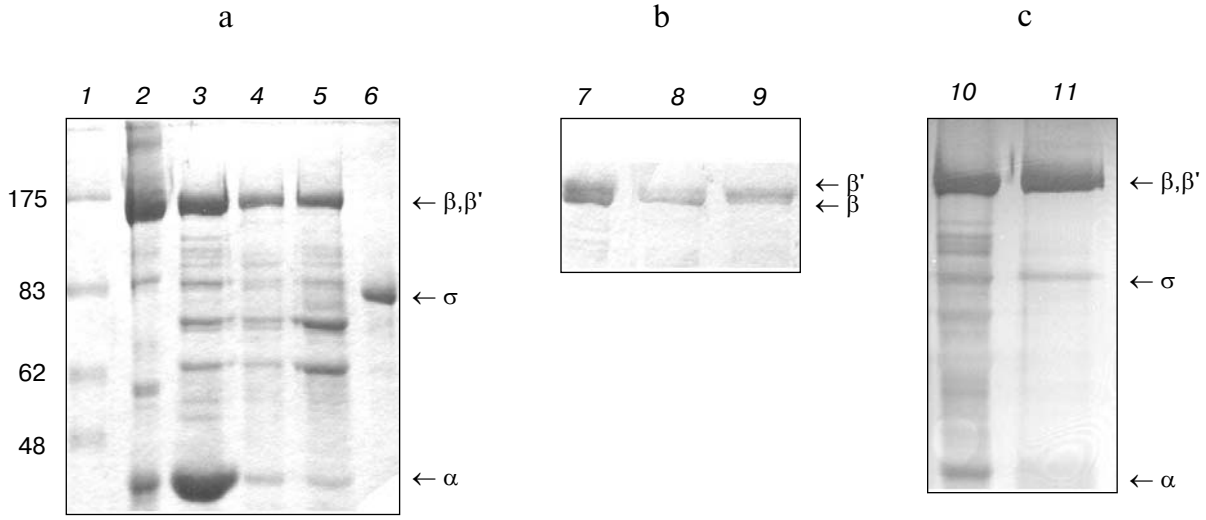

Fig. 3. Electrophoretic analysis of proteins in 8% SDS-polyacrylamide gel. a) Protein preparations isolated on a chitin-containing column from cleared homogenates of cells with plasmids pC4-a (*3*), pC4-b (*4*), pC4-c (*5*), and pC4-d (*6*) expressing the α, β, β′, and σ subunits of RNAP with intein–CBD domain. b) More prolonged electrophoresis for separation of β and β′ subunits of RNAP in the preparations shown in lanes *3* (*7*), *4* (*8*), and *5* (*9*). c) Comparative analysis of RNAP preparation from cells with pC4-a plasmid concentrated by ultrafiltration before (*10*) and after (*11*) additional purification on heparin-agarose. Lanes: *1*) protein markers; *2*) commercial RNAP holoenzyme preparation. Arrows on the side, location of RNAP subunits; figures on the left, molecular weights of protein markers, kD.

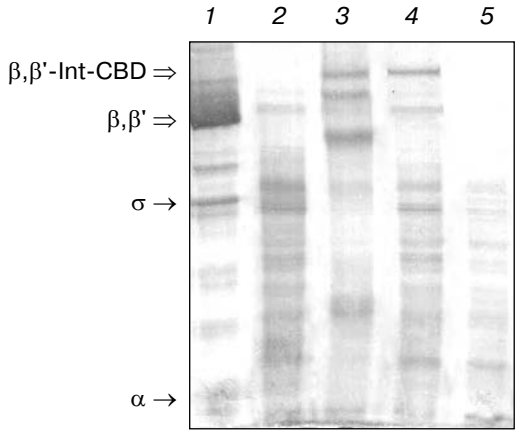

Fig. 4. Electrophoretic analysis (8% SDS-PAGE) of protein preparations isolated from debris of cells containing the following plasmids: pC4-a (*2*), pC4-b (*3*), pC4-c (*4*), and pUC18 (*5*); *1*) commercial RNAP holoenzyme preparation. Arrows on the side, location of RNAP subunits.

and β′ subunits with intein–CBD domain attached (Fig. 4, lanes *3* and *4*).

However, significant amounts of proteins able to bind specifically to chitin-containing resin were found in homogenates after centrifugation (cleared homogenates) of cells with pC4-b and pC4-c plasmids. It turned out that in both cases IMPACT technology extracts all four *E. coli* RNAP subunits, α, β, β′, and σ, from cleared cell homogenates, where the molar ratio corresponds to holoenzyme (Fig. 3, lanes *4* and *5*). In both cases there was no very high excess of the corresponding subunit (β or β′; Fig. 3, lanes *8* or *9*) heterologously expressed with intein–CBD domain removed during the isolation procedure, as, for instance, in the case of α subunit (Fig. 3, lane *3*).

These data indicate that all three major RNAP subunits (α, β, and β′) with intein–CBD domain at the C-terminus of the polypeptide chain, which are heterologously expressed from artificial plasmid operons, are able to specifically interact with corresponding native subunits directly in cell cytoplasm, forming protein complexes resembling that of native RNAP. Considering the conditions for complex assembly (*in vivo*) and mild isolation conditions (adsorption on chitin-containing resin and detachment by DTT), one could expect that the corresponding RNAP preparations would display enzymatic activity. To confirm this expectation we studied the ability of the isolated proteins to form specific (open) complexes with promoter-containing DNA and perform specific promoter-dependent transcription initiation. Protein preparations obtained from the three cell lines transformed with pC4-a, pC4-b, and pC4-c plasmids were used in these experiments.

The ability of isolated RNAP preparations to form open promoter-specific complexes was studied by the gel retardation method. For this purpose, incomplete synthetic DNA duplex 66_1:41_2 was used (Scheme 1). The duplex contained two consensus elements in the promoter region near –35 (TTGACA) and –10 (TATAAT) nucleotide bases separated by a 17 bp spacer region. It is known from the literature [26] that such duplexes with a single-stranded region near the –10 nucleotide base (Pribnow box) are able to form strong heparin-resistant complexes with RNAP holoenzyme, imitating open promoter-specific complexes. Preliminary data demonstrated that the half-dissociation time for the complex of RNAP holoenzyme with 66_1:41_2 duplex is over 2 h. The reaction mixtures containing 5′-[^{32}P]p66_1:41_2 DNA duplex and RNAP protein preparation being studied were incubated at 37°C, and the interaction products were analyzed by gel electrophoresis. To reveal promoter-specific complexes resistant to polyanions, heparin was added to the reaction mixtures. In some experiments, RNAP preparations were additionally saturated with σ subunit. The results of the experiments are shown in Fig. 5. As seen from the figure, in all cases 5′-[^{32}P]p66_1:41_2 DNA duplex forms complexes with isolated proteins whose electrophoretic mobility corresponds to the mobility of the complexes with commercial RNAP holoenzyme preparation. Besides, the extent of binding is practically unchanged both upon addition of heparin or saturation of preparations with σ subunit. These results indicate the presence of correctly assembled RNAP molecule (represented by a significant amount of holoenzyme) in protein preparations isolated from cell cytoplasm according to the IMPACT technology.

Examination of transcriptional activity of isolated preparations was performed using a template with two divergent promoters (the regions near –35 and –10 nucleotide bases are underlined; arrows show transcription initiation sites) (Scheme 2).

It has been previously demonstrated that RNAP holoenzyme generated two transcripts of 84 and 88 nucleotide bases. In all cases, the mixture of template with protein preparation under investigation was preincubated at 37°C, and transcription was initiated by addition of ribonucleoside triphosphates in the presence or

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                -35                  -10
5′-CGTCTTCAAGAATTCCCTTGACACTTACTCATCCGGATCCTATAATGGGGGATCCGTCGACCTGCA-3′
3′-GCAGAAGTTCTTAAGGGAACTGTGAATGAGTAGGCCTAGGA-5′
```

Scheme 1

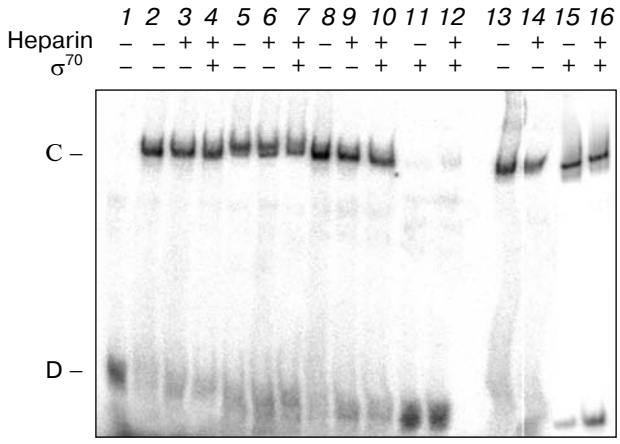
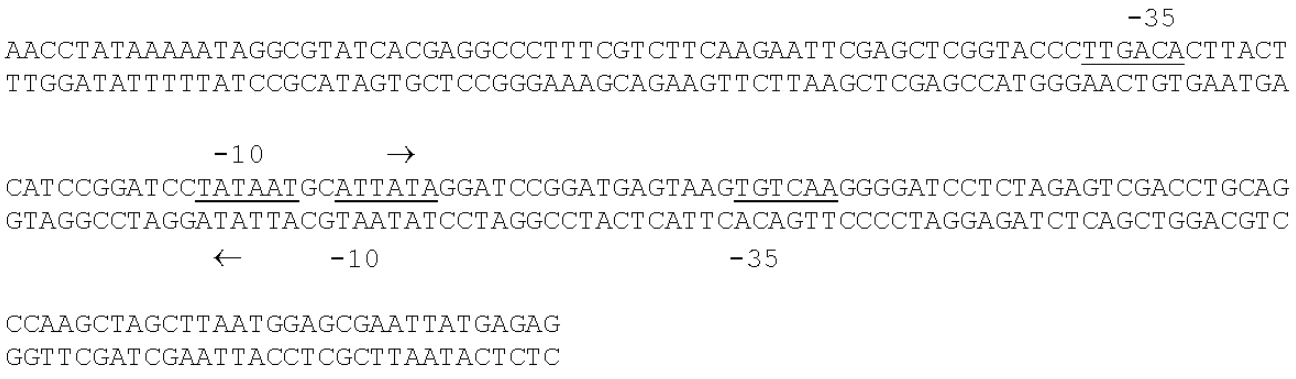


Fig. 5. Radioautograph of electrophoretic separation (4% nondenaturing polyacrylamide gel) of the complexes of 5′-[^{32}P]p66$_1$:41$_2$ DNA duplex with protein preparations from cells with plasmids pC4-a (*2-4*), pC4-b (*5-7*), pC4-c (*8-10*), pC4-d (*11*, *12*), as well as with commercial preparations of RNAP holoenzyme (*13*, *14*) and core enzyme (*15*, *16*); *1*) initial 5′-[^{32}P]p66$_1$:41$_2$ duplex. The location of 5′-[^{32}P]p66$_1$:41$_2$ duplex (D) and its complex with RNAP (C) is shown on the side. Signs "+" and "–" denote the conditions for complex formation: preliminary saturation of RNAP with σ subunit and/or addition of heparin to the reaction mixture.

absence of heparin. In some experiments excess of σ subunit was previously added to the preparations under investigation. The reaction mixtures were analyzed by electrophoresis; the appearance of characteristic 84- and 88-mer polynucleotides indicated the progress of promoter-dependent reaction. The experimental results presented in Fig. 6 provide evidence that all preparations isolated according to the IMPACT technology display a profound ability to initiate transcription specifically, i.e. contain functionally active RNAP holoenzyme. It should be noted that addition of σ subunit or carrying out the reaction in the presence of heparin in the case of RNAP preparations isolated from cells with pC4-a and pC4-c plasmids do not change the general transcription pattern, whereas preliminary saturation with σ subunit in the case of preparation isolated from cells with pC4-b plasmid significantly increases the extent of specific transcription (compare lanes *9*, *10*, and *11* in Fig. 6). Moreover, relatively lower initial content of σ subunit in this preparation is also noticeable during its direct electrophoretic analysis by SDS-PAGE (Fig. 3, lane *4*). This feature was observed in a number of experiments. It can be assumed that during assembly of RNAP *in vivo* with participation of "modified" β subunit, the intein–CBD domain attached at the C-terminus of polypeptide chain hinders the interaction between σ subunit and the core enzyme to larger extent than in the case of modified α and β′ subunits, which apparently decreases the stability of the complex between σ subunit and the core enzyme. It results in a partial loss of σ subunit during the first stages (before DTT treatment) of the isolation procedure. This assumption does not contradict the generally accepted structural model of RNAP complex [27-33], according to which some contacts of σ subunit with the core enzyme are located in proximity of the C-terminus of the β subunit polypeptide chain [34, 35]. In contrast, C-terminal regions of the two α subunits and β′ subunit are located on the surface of the enzyme molecule and are not involved in interactions important for enzyme assembly. It is probable that the development and use of larger auxiliary intein-containing domains will allow isolating RNAP preparation free of σ subunit (core enzyme).

The preparation of σ subunit isolated by IMPACT technology has been tested for its enzymatic activity in separate experiments. The results of these experiments are presented in Figs. 5 and 6. The absence of specific complexes with 5′-[^{32}P]p66$_1$:41$_2$ DNA duplex (Fig. 5, lanes *11* and *12*) and transcription products (Fig. 6, lanes *15* and *16*) indicates the complete absence of endogenous RNAP impurities in the studied preparation. At the same time, addition of the same σ subunit preparation to RNAP core enzyme (commercial preparation) results in the assembly of functionally active holoenzyme, and appearance of ability of the latter to form a specific complex with promoter (Fig. 5, lanes *15* and *16*) as well as initiate promoter-specific transcription (Fig. 6, lanes *2-4*).



Scheme 2

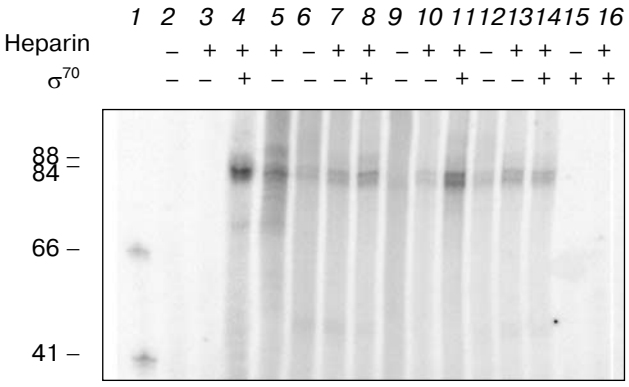


Fig. 6. Radioautograph of electrophoretic separation (8% denaturing polyacrylamide gel) of *in vitro* transcription products on a template with two divergent promoters. Reactions were performed after saturation with σ subunit (+) or without it (–) in the presence (+) or absence (–) of heparin using commercial preparations of RNAP core enzyme (*2-4*) and holoenzyme (*5*), as well as protein preparations from cells containing plasmids pC4-a (*6-8*), pC4-b (*9-11*), pC4-c (*12-14*), and pC4-d (*15*, *16*); *1*) oligodeoxyribonucleotides 5′-[^{32}P]p41$_2$ and 5′-[^{32}P]p66$_1$. Figures on the left, base number in ^{32}P-labeled polynucleotides.

Therefore, it turned out that it is possible to produce appreciable amounts of functionally active RNAP formed in completely native conditions using α, β, or β′ subunits with attached technological module obtained by IMPACT technology. However, it should be noted that despite the major bands corresponding to α, β, β′, and σ subunits, there are also bands of minor impurities (not removed by additional washes of the affinity column including 2% Triton X-100 solution, 2 M urea, 20% ethanol, and acetonitrile) observed upon electrophoretic analysis of the preparations (Fig. 5, lanes *3-5*, *10*). It is most likely that along with RNAP, some other proteins forming sufficiently stable complexes with the enzyme are also isolated under very mild purification conditions according to the IMPACT technology. Enzyme preparation can be purified until virtually homogeneous state using standard techniques, for instance, chromatography on heparin-agarose (Fig. 3, lane *11*). Yields of the purified preparations vary and may reach 0.5-1.0 mg per liter of cell culture, thus corresponding to normal level (for example, 0.25 mg [18] or up to 1 mg [20] per liter) of production of heterologously expressed RNAP.

It should be mentioned that the possibility of *in vivo* RNAP assembly with participation of α and β′ subunits containing the relatively small hexahistidine module has been described in literature [18, 36]. One study [20] also reports *in vivo* production of RNAP (core enzyme) with β′ subunit containing the intein–CBD domain; however, RNAP was assembled from subunits heterologously expressed from the single plasmid operon.

In our work we succeeded in obtaining functionally active RNAP by heterologous expression of only one of α, β, or β′ with attached intein–CBD domain. This way opens the possibility of *in vivo* assembly of RNAP under native conditions and rapid isolation of its any mutant forms completely free of admixtures of the native enzyme.

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