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EXPRESSION OF THE N-TERMINAL SEGMENT OF QBRN-2 IN *E. COLI* AND TIPS ON PREPARATION OF A RECOMBINANT PROTEIN

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Abstract: POU box gene qBrn-2 exhibits restricted expression pattern in quail neurogenesis and myogenesis. Here we report isolation of a N-terminal segment (P207) of qBrn-2 expressed in *E. coli* and tips on preparation of a recombinant protein. Recombinant P207 was purified and migrated as a single band on SDS-PAGE with the same apparent molecular mass as predicted. In the expression of P207 by pET 3b vector, IPTG concentration for induction greatly affected the expression level. Further, fusion domain at N-terminus seemed to change the stability of P207. Those results suggest some useful tips on preparation of a recombinant protein.

Key words: POU, recombinant protein, expression, *E. coli*, degradation

Introduction

POU domain is a bipartite DNA-binding domain, consisting of POU specific domain and POU homeodomain tethered by a variable linker. Ever since its discovery, POU domain proteins from diverse range of species have been identified and grouped into six classes based on the amino acid sequence of their POU domains and conservation of the variable linker. The spatiotemporal expression pattern of POU domain proteins and function analysis have demonstrated that POU proteins play critical roles in embryogenesis, both early and terminal differentiation of specific cell types [1-4]. qBrn-2, cloned from a quail cDNA library, belongs to POU transcription factor family [5]. It can bind to corticotropin-releasing hormone

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Genbank accession number: AF091043.

Abbreviations used: *E. coli: Escherichia coli*; GST, glutathione *S*-transferase; aa, amino acid; IPTG, isopropyl *b*-D-thiogalactopyranoside; LB, Luria broth; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate.

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II site and exhibits restricted expression pattern in quail neurogenesis and somitic myogenesis, suggesting that qBrn-2 might play important roles in those processes [6]. As the first step to prepare a specific antibody against qBrn-2, we expressed the N-terminal segment (P207) of qBrn-2 in *E. coli*. Here, we report the expression of recombinant P2-7 and some tips on preparation of a recombinant protein.

Materials and Methods

Bacterial strains and plasmids

qBrn-2 was previously cloned from E5 quail cDNA library in this lab and deposited in Genbank. Glutathione Sepharose, Chelating Sepharose Fast Flow, pGEX 4T-1 and bacterial strain BL21 were from Pharmacia. pET 3b, pET 28a, and bacterial strain BL21(DE3) were from Novagen. *Bam*HI linker and enzymes used for molecular biology were from Promega.

Construction of plasmids

The *Hin*fI-*Sma*I fragment of qBrn-2 cDNA (from – 4 to +621) was first filled by Klenow DNA polymerase and then ligased with *Bam*HI linker (12 mer). After *Bam*HI digestion, the fragment was in frame cloned into the *Bam*HI site of pET 3b with correct orientation, resulting pET 3b 207. Sequence at the junction was confirmed by DNA sequencing. pHis 207 was constructed by replacing the *Xba*I-*Nde*I fragment of pET 3b 207 with the *Xba*I-*Nde*I fragment of pET 28a which encodes a His-tag. PGST 207 was constructed by insertion of the *Sma*I-*Sma*I fragment of qBrn-2 cDNA (from –37 to +621) into the *Sma*I site of pGEX 4T-I with the correct reading frame and orientation. For pET 3b 207 and pHis 207, bacterial strain BL21(DE3) was used as the host [7]. For pGST 207, bacterial strain BL21 was used [8].

Examination of expression in E. coli

Expression of recombinant proteins in *E. coli* was examined as described before [7]. Briefly, 1.0 ml of culture at appropriate times was collected and centrifuged. The cell pellets were suspended in 200 µl of sample buffer [50 mM Tris-HCl, pH 6.8, 2 mM Na_3EDTA, 1% (w/v) sodium dodecyl sulphate, 1% (v/v) mercaptoethanol, 8% (v/v) glycerol, 0.025% (v/v) bromophenol blue], boiled for 2-3 min, and the supernatant was directly loaded to 10% SDS-PAE for protein induction or to 1% agarose gel and visualized by ethidium bromide for mRNA induction.

Purification of recombinant-protein

An overnight bacterial culture was diluted with LB medium (1 : 50, v/v) and cultured at 37°C to an OD_{600}=0.6 ~ 0.8. Overexpression was induced by adding IPTG at a proper concentration. After further 3-h growth at 37°C, the bacteria were harvested and resuspended in PBS containing 1 µg/ml of aprotinin and 100 µg/ml of PMSF, and sonicated in ice for several bursts. Then Triton X-100 was added (1% as a final concentration) with gentle shaking for 30 min. Supernatant was

obtained by centrifugation at 4°C. Peptide His-P207 or GST-207 was purified by Ni^{++} -chelating Sepharose column or glutathione-Sepharose column according to the instructions of manufacture, respectively.

Overexpression and purification were analyzed by 10% SDS-PAE (Laemmli gels) and Molecular Analyst ™/PC software. Protein concentration was determined by Lowry method.

Results

Construction of expression plasmids

The structure of qBrn-2 cDNA and the expression plasmids were shown in Fig. 1. The clone qBrn-2 had on open reading frame encoding 431 amino acid residues and the POU domain was situated at its C-terminues. In pET 3b 207, P207 was expressed as a fusion peptide with a short T7 –tag (12 aa) at its N-terminus under the control of T7 10 promoter and 17 transcription terminator. To facilitate purification of the recombinant peptide, pHis-207 was constructed by modification of pET 3b 207. In pHis 207, a His-tag was added to the N-terminus of T7-tag. In pGST 207, P207 was expressed as a fusion peptide with GST domain at its N-terminus under the control of Tac promoter.

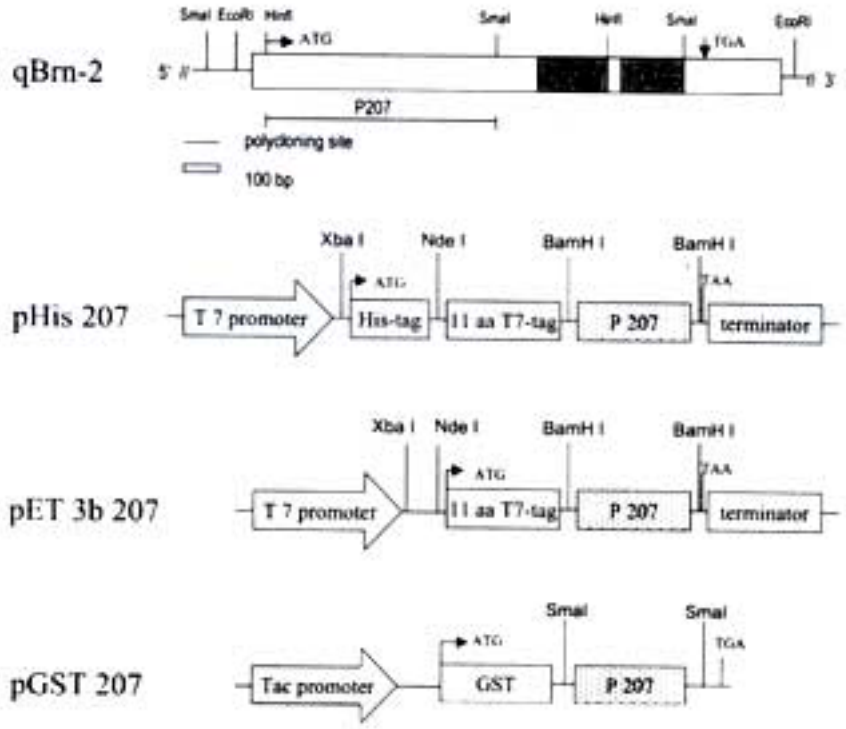

Fig. 1: Structure of qBrn-2 cDNA and expression plasmids. ATG: initial codon of translation; TAA, TGA: stop codons of translation; P207: the N-terminal segment of qBrn-2 (amino acid residues 1-207); POU_{SD}: POU specific domain; POU_{HD}: POU homeodomain; GST: glutathione *S*-transferase.

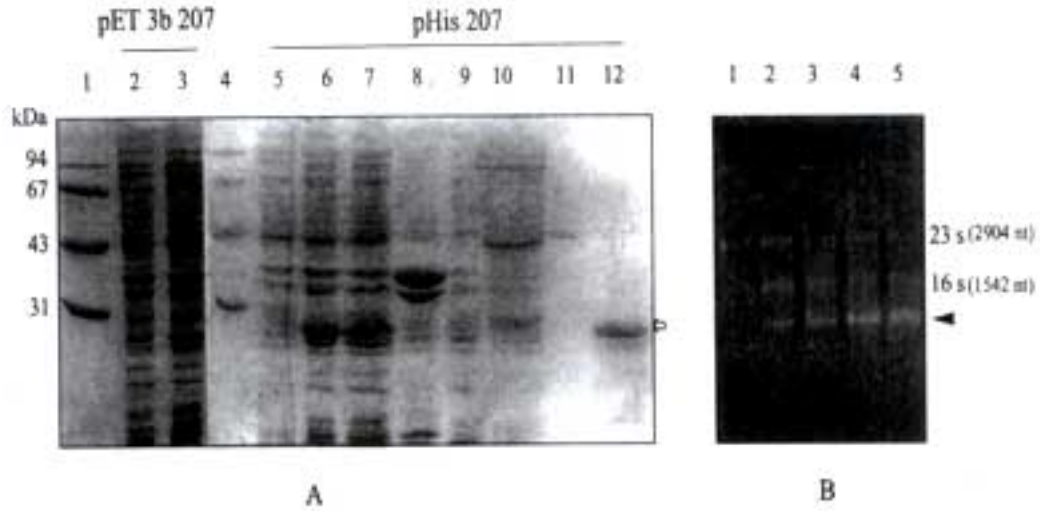

Fig. 2: Overexpression and purification of P207 by pET vectors. (A) Overexpression of pET 3b 207 and pHis 207. The band corresponding to the purified His-P207 was indicated by an arrow. Lane 1: protein molecular mass marker, lanes 2-3: total bacterial extracts of BL21(DE3) harboring pET 3b 207 at the time before and 3 h after IPTG induction (0.05 mM as a final concentration), respectively; lane 4: protein molecular mass marker, lanes 5-6: total bacterial extracts of BL21(DE3) harboring pHis 207 before and after IPTG induction; lanes 7-12: samples at different steps of His-P207 purification: supernatant of sonicated lysate, precipitate of sonicated lysate, flow through, 20 mM imidazole wash, 50 mM imidazole wash and 200 mM imidazole elution, respectively. (B) Induction of mRNA by IPTG (0.05 mM as a final concentration). The band corresponding to the induced mRNA was indicated by an arrow. Lane 1: bacterial extract immediately before IPTG induction; Lanes 2-5: extracts 1~4h after IPTG induction, respectively.

Overexpression and purification of P207 fusions

Overexpression of P207 fusion by pET 3b 207 was performed as described in methods. The result showed that, after 0.05 mM I PTG induction, a dark extra protein band of desired size was observed, indicating that intact P207 fusion was overexpressed (Figure 2A, lane 2 and lane 3). Interestingly, the expression level was obviously affected by the concentration of IPTG. When 0.8 mM IPTG was used, almost no protein was induced although large amounts of mRNA accumulated in bacteria (data not shown). However, when the concentration decreased, the expression level increased. The optimal concentration of IPTG was approximately 0.05 mM.

We also examined the accumulation of mRNA in the host (Fig. 2B). After addition of IPTG (0.05 mM as a final concentration) large amounts of new mRNA were induced, and the calculated size was the same as predicted with 23s (2904 nucleotides) and 16s (1542 nucleotides) bacterial rRNA as standard [9]. The mRNA was stable for an additional five hours in culture.

To facilitate purification of the recombinant peptide, a His-tag was added to the N-terminus of the short T7-tag as represented by pHis 207. The expression level of pHis 207 was almost the same as that of pET 3b 207 (Fig. 2A, lane 3 and lane 6). The bands corresponding to the His-P207 in whole cell extracts and the supernatant of sonicated lysate showed

almost the same density (Fig. 2A, lane 6 and lane 7), indicating that His-P207 was highly expressed in soluble form. His-P207 was purified to homogeneity in one-step. By passing a Ni^{++} - chelating Sepharose column, most contaminant proteins were washed off by 50 mM imidazole. Then His-P207 was eluted by 200 mM imidazole. The eluted fraction migrated as a single band on SDS-PAGE with the same apparent molecular mass as predicted (26 kDa), and its purity was 92% revealed by Molecular Analyst^{TM}/PC software. Typically, 1-liter culture produced 5-10 mg of purified His-207.

In contrast, expression of GST-P207 was quite different. The sample purified by glutathione-Sepharose appeared to be several bands on SDS-PAE (Fig. 3A). The protein of the desired size only showed as a minor band, while the major proteins were around 31 kDa. Obviously, the expressed recombinant peptide was truncated from its C-terminus. Changes in induction time or culture temperature did not decrease the protein degradation. The degradation seemed to occur before cell lysis, since addition of proteases inhibitors (aprotinin and PMSF) to the lysis buffer gave little effect.

To investigate reasons for the degradation, we also examined the accumulation of mRNA in the host. Agarose gel electrophoresis showed that, after IPTG induction, two intact bands of mRNA (6.0 kb and 4.7 kb, respectively) were induced (Fig. 3B), suggesting that the truncation of GST-P207 was not caused by degradation of mRNA. The two bands might represented transcripts which ended at different sites.

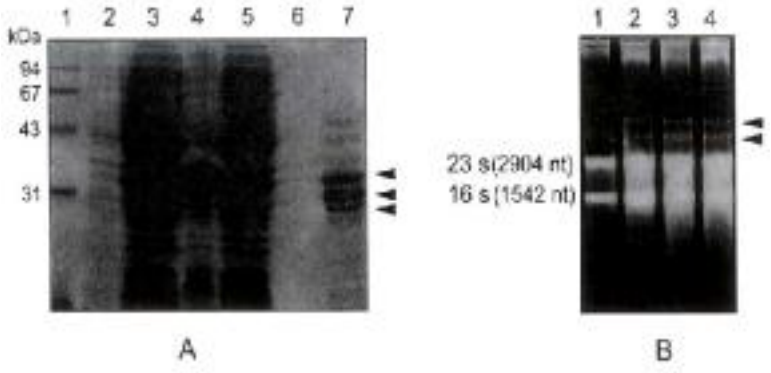

Fig. 3: Instability of GST-207. (A) Overexpression and purification of GST-P207. The bands corresponding to the degraded proteins were indicated by arrows. Lane 1: protein molecular mass marker; lane 2: total bacterial extract before IPTG induction; lanes 3-7: samples at different steps of GST-P207 purification: supernatant of sonicated lysate, precipitate of sonicated lysate, flow through of glutathione-Sepharose column, the final PBS wash, and elution by 10 mM reduced glutathione in 50 mM Tris-HCl (pH 8.0), respectively. (B) Accumulation of induced mRNA. The bands corresponding to two transcripts with different length were indicated by arrow. Lane 1: bacterial extract at the time immediately before IPTG induction; lanes 2-4: bacterial extracts 1,2, and 3 h after IPTG induction, respectively.

Discussion

Many vectors with *E. coli* as host organism have been developed to express proteins from cloned genes. Despite hundreds of successful examples, expression of a new gene occasionally present problems such as very low expression

level or degradation of the recombinant protein. Those questions are more obvious for those genes that are involved in developmental regulation during embryogenesis.

In this text we report purification of P207 expressed in *E. coli*. The recombinant peptide has been successfully used in preparation of a specific antibody against qBrn-2 protein [6]. In the expression of P207, we found that the expression level depended on some conditions, which might be useful in expression of other genes.

In pET/BL21(D3) system, the concentration of IPTG obviously affected the expression level. When 0.8 mM IPTG was used, almost no P207 fusion was produced in the bacterium BL21(DE3) harboring pET 3b 207 although large amounts of mRNA were induced and accumulated. However, when 0.05 mM IPTG (much low than the commonly used concentration) was used, moderate high level of P207 fusion protein was produced. The mechanisms for the low protein production at higher IPTG concentration are still not known. However, it is reasonable that the optimal concentration of IPTG varies in different applications. Further, P207 is segment of a transcription factor and might somehow interfere the expression machinery of bacteria. Lastly unfavorable distributions of rate codons, relatively high levels of translational frameshifting, interfering structures in the mRNA might also account for the above phenomenon.

Another interesting result was that the stability of P207 was quite different when it was fused to different domains. In one system intact fusion peptide was produced, while in another system fusion peptide obviously degraded in bacteria. It is interesting to investigate the reasons for the phenomenon. In the two systems, genetic backgrounds of the hosts (BL21 and BL21(DE3)) were almost the same and might not make stability difference of the recombinant proteins. And, in the two systems, intact mRNA accumulated and should not produce truncated peptide if full-length mRNA was translated. The major difference in the two systems was the fusion domain at the N-terminus of P207. A probable reason for the instability of GST-P207 was that the GST domain changed the conformation of P207 and made its interior sensitive sites to be accessible to the proteases in *E. coli*. Hence, special attention should be paid to the possible interactions between the fusion domain and the target protein when a cloned gene is expressed with a fusion peptide.

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