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Recombinant expression, purification and preliminary characterization of the mRNA export factor MEX67 of *Saccharomyces cerevisiae*

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ABSTRACT

The nuclear export of macromolecules is facilitated by the nuclear pore complexes (NPCs), embedded in the nuclear envelope and consists of multi-protein complexes. MEX67 is one of the nuclear export factor responsible for the transport of the majority of cellular mRNAs from the nucleus to the cytoplasm. The mechanism of mRNA transport through NPCs is unclear due to the unavailability of structures and the known interacting partners of MEX67. The *mex67* gene was cloned in pQE30A and was expressed in *Escherichia coli*. A strategy has been developed to purify the insoluble MEX67 using a nickel affinity column with chelating Sepharose fast flow media, after solubilizing with sodium lauroyl sarcosinate (Sarkosyl). The IMAC purified recombinant MEX67 was further purified using SEC to apparent homogeneity (~8 mg/L). Following SEC, MEX67 was stable and observed to be a 67 kDa monomeric protein as determined by PAGE and the size exclusion chromatography. The availability of large quantities of the protein will help in its biochemical and biophysical characterization, which may lead to the identification of new interaction partners of MEX67 or MEX67 complex.

Introduction

The nuclear export of macromolecules between the nucleus and the cytoplasm occurs through nuclear pore complexes (NPCs)[1], which are large multi-protein complexes embedded in the nuclear envelope. Mostly, soluble transport receptors are responsible for the translocation of proteins through the NPC [1]. NPCs are believed to be composed of 30–40 different proteins (nucleoporins), and many of these have now been cloned, sequenced and expressed. MEX67 is one of the nuclear mRNA export factors and is responsible for the export of a majority of cellular mRNAs to the cytoplasm. These nuclear export factors (NXF) are a group of evolutionarily conserved proteins [2]. Several NXF genes have been identified in humans (termed as NXF1–6), *Drosophila melanogaster* (Dm NXF1–4) and *Caenorhabditis elegans* (Ce NXF1–2) while only one NXF isoform (MEX67) exists in *Saccharomyces cerevisiae* [3–5]. The two best-characterized NXFs are MEX67 (*S. cerevisiae*) and NXF1 or TAP (human). MEX67 in association with Mtr2 constitute a novel mRNA export complex where mRNA binds to MEX67 and Mtr2 interacts with nuclear pores [6]. Mutation studies show that the dissociation of MEX67 from the pores result in a strong inhibition of mRNA export, indicating its importance in mRNA transport [6]. Many proteins have been reported to interact physically with MEX67 [7,8]. Genetic interaction to MEX67 or MEX67 complex has also been reported [6,7,9,10]. The complete interacting partners of MEX67 have not been elucidated due to the unavailability of soluble and purified MEX67. In vitro studies need purified MEX67 that can be used for the identification of the total set of protein/complexes associated with MEX67.

Protein expression in *Escherichia coli* provides the ability to obtain large amounts of recombinant proteins rapidly. pQE30 is one of the most extensively used vector for the expression and purification of recombinant proteins. Although the expression and production of many recombinant proteins are well established, certain obstacles can however affect the expression and production of soluble full-length recombinant proteins. Thus, the limiting factors in the recombinant protein production include no expression, insoluble protein production and improper purification. Osmotic stress for enhancing the expression level and mild detergent for solubility are some strategies for large-scale recombinant protein production [11–17]. In the present work, we report cloning, successful overexpression and purification of MEX67. We mainly focus

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[1] *Abbreviations used:* SEC, size exclusion chromatography; Ni–NTA, nickel–nitrilotriacetic acid; IMAC, immobilized metal affinity chromatography; NPCs, nuclear pore complexes; NXFs, nuclear export factors; Sarkosyl, sodium lauroyl sarcosinate.

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on the optimization of expression and solubility of MEX67 so that further studies can reveal its novel interacting partners.

Materials and methods

The molecular biology kits and nickel-nitrilotriacetic acid (Ni–NTA) agarose matrix were purchased from Qiagen, CA, USA. The deoxynucleotide triphosphates (dNTPs) and enzymes were purchased from New England Biolabs, MA, USA. All other reagents and chemicals were purchased either from the Sigma–Aldrich Chemical Company, St. Louis, MO, USA or Sisco Research Laboratories, Mumbai, India and were of the highest purity available. Bacterial culture media were purchased from Himedia Laboratories, Mumbai, India.

Isolation of S. cerevisiae genomic DNA

A single yeast colony was picked from the yeast extract peptone dextrose (YPD) agar plate and inoculated into 2 mL of rich media (YPD: 1% yeast extract, 2% peptone, 2% dextrose). It was grown in an incubator shaker at 30 °C for approximately 24 h. Next day, the culture was pelleted in a microfuge tube, and the pellet was resuspended in 200 μL of lysis buffer containing 10 mM Tris–HCl (pH 8.0), 100 mM NaCl, 2% Triton X-100, 1% sodium dodecyl sulfate (SDS) and 1 mM ethylenediaminetetraacetic acid (EDTA) [17]. The tube was placed in a −80 °C freezer for 2 min and then immersed in a 95 °C water bath for 1 min to freeze thaw quickly. The process was repeated twice. The tube was then vortexed vigorously for 10 s. Then 200 μL of phenol–chloroform (1:1) was added, and the tube was again vortexed for 1 min and centrifuged for 10 min at 13,000 rpm at room temperature. The upper aqueous layer was transferred to a microfuge tube containing 300 μL of ice-chilled 100% ethanol. The sample was allowed to precipitate for 5 min at room temperature and then centrifuged at 13,000 rpm for 5 min. The supernatant was decanted out, and the DNA pellet was washed with 500 μL of 70% ethanol and kept in a dry bath at 60 °C for 5 min for drying. The DNA was then resuspended in 20 μL of syringe-filtered triple-distilled water. The concentration of the isolated DNA was determined using Nanovue (GE Healthcare Biosciences, USA).

Cloning of mex67

Primers were designed based on the *mex67* sequence from the *S. cerevisiae* Genome Database. Synthetic oligonucleotides were purchased from GCC Biotech (Kolkata, India). The gene coding for full-length *mex67* of size 1.8 Kb was amplified using the gene specific primer set forward primer (5′-GGATCCATGAGCGGATTTCACAATG-3′) containing a BamHI site and the reverse primer (5′-AAGCTT-GAACTGCACAAATGCTTC-3′) containing a HindIII site. The polymerase chain reaction (PCR) was performed using Phusion High-Fidelity DNA Polymerase with the following cycle parameters: initial denaturation temperature of 97 °C for 10 s, 30 cycles of 97 °C for 10 s, 55 °C for 15 s and 72 °C for 1 min followed by a final extension of 72 °C for 5 min. The PCR amplicon was ligated into the pSK^+ vector. The positive clone pSK^+-*mex67* encoding plasmid was confirmed by sequencing. The plasmid was then digested with BamHI and HindIII, and the product was purified using agarose gel electrophoresis. It was then ligated to BamHI and HindIII digested pQE30 vector to generate the recombinant construct pQE30-*mex67*. The recombinant plasmid was transformed into chemically competent SG13009 expression host cells by the heat shock method and then spread onto agar plate containing ampicillin and kanamycin to allow selection of colonies that successfully incorporated the plasmids. Plasmid DNA extraction was performed using the QIAprep Midiprep plasmid purification kit (Qiagen, USA). Nucleotide sequencing was carried out in the Xcleries lab (Gujarat, India).

Optimization of recombinant MEX67 expression in presence of osmolytes

A single colony was picked from the transformed lysogeny broth (LB) agar plate and was inoculated into 5 mL LB broth containing 100 μg/mL ampicillin and 50 μg/mL kanamycin in an autoclaved culture vial. It was incubated at 37 °C for overnight with continuous shaking. Next morning, 50 μL of the primary culture was inoculated in five 5 mL LB broth supplemented with the above mentioned antibiotics and osmolytes-glucose (100 mM), sorbitol (100 mM), mannitol (100 mM) and 1% ethanol separately. One set was inoculated, and the other was used as un-induced control. All cultures were incubated at 37 °C with shaking until the OD_{600} reached 0.5. After induction with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), the culture vials were incubated at 23 °C with continuous shaking for overnight. Next day, the cells were harvested by centrifugation at 6000 rpm for 15 min and resuspended in lysis buffer (50 mM Tris, pH 8.0, 300 mM NaCl containing protease inhibitor cocktail), sonicated and then analyzed by SDS–polyacrylamide gel electrophoresis (PAGE).

Optimization of recombinant MEX67 expression using different concentrations of ethanol and variable temperature

A single colony was picked from the transformed LB agar plate and was inoculated into 5 mL LB broth containing 100 μg/mL ampicillin and 50 μg/mL kanamycin in an autoclaved culture vial. It was incubated at 37 °C with continuous shaking for overnight. Next morning, 50 μL of the primary culture was inoculated in two sets of five 5 ml LB broth supplemented with the above mentioned antibiotics and different concentration ethanol (0%, 0.5%, 1%, 2% and 3%) in autoclaved culture vials. Another 5 mL LB broth culture tube containing the above-mentioned antibiotics were inoculated with the primary culture and was used as an un-induced control. All cultures were incubated at 37 °C with shaking until the OD reached 0.5. After induction with 0.5 mM IPTG, one set of five culture vials were incubated at 37 °C with shaking for 4 h while another set of five culture vials were incubated at 23 °C with shaking for overnight. Next day, the cells were harvested by centrifugation at 6000 rpm for 15 min and resuspended in lysis buffer (50 mM Tris, pH 8.0, 300 mM NaCl containing protease inhibitor cocktail), sonicated and then tested by SDS–PAGE for comparative study of protein expression level in the presence of different concentrations of ethanol and temperature difference.

Large-scale culture and expression of recombinant MEX67 using ethanol

After optimizing the expression level, the primary culture of MEX67 overproducing strain-SG13009-pQE30-*mex67* was inoculated in 400 mL sterile LB broth in 1:50 ratio supplemented with 100 μg/mL ampicillin, 50 μg/mL kanamycin and 3% ethanol (v/v). Then it was incubated at 37 °C with continuous shaking until the OD_{600} reached 0.5. Another 5 mL LB broth culture tube containing the above-mentioned antibiotics was inoculated with the primary culture and was kept as an un-induced control. Once the OD_{600} reached 0.5, the culture was induced with 0.5 mM IPTG and incubated in a shaking incubator. The induction time was 12 h at 23 °C. Next day, the cells were harvested by centrifugation at 4 °C, and the pellet was resuspended in 20 mL of 50 mM Tris, pH 8.0, 300 mM NaCl containing protease inhibitor cocktail.