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Cloning and expression of *UbiA* human gene using innovative methodologies for recombinant protein production in PUAST vector

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Abstract

Introduction: A systematic library of *Gal4/UAS*-regulated transgenes has proven to be a powerful genetic system for identifying genes and defining pathways in development. This system offers valuable insights that highlight the evolutionary conservation between animals and humans.
Objectives: The objective of this study was to clone, express, and characterize the *UbiA* gene. The research presents a highly effective method for cloning genes, using the *UbiA*-pcDNA3 gene as a model for mammalian cloning. These genes were then integrated into the PUAST vector of *Drosophila*, an expression vector and eukaryotic cell system commonly used for producing recombinant proteins.
Materials and Methods: *UbiA* was isolated from human cells, and complementary DNA was synthesized. An oligonucleotide primer pair was designed based on the *UbiA* gene sequence, incorporating XhoI and XbaI restriction sites at the 5´ end of the forward and reverse primers, respectively. The *UbiA* gene was then amplified by PCR, cloned into the pcDNA3 plasmid, and the resulting recombinant plasmid was sequenced. Subsequently, the gene was sub cloned into the PUAST vector and expressed in S2 cells as a eukaryotic cell system. Protein determination and verification were conducted through western blotting techniques.
Results: Confirmation of *UbiA* gene cloning into the PUAST vector was achieved through colony-PCR and digestion by enzymes. Cloning and sub cloning techniques validated by enzymatic digestion, along with gene sequencing. The identity between cloned *UbiA* gene and the identical gene exhibited 99%. We revealed a singular band purified protein through western blotting with 60 kDa size.
Conclusion: More protein synthesis of the *UbiA* gene can be achieved by using the eukaryotic expression system provided by the PUAST vector. This technique has been proven to be a suitable platform and can be instrumental in various applications such as therapeutics, pharmacology, and vaccine development.

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Introduction

Model organisms like S2 cell, have become instrumental in understanding the complexities of functional genomics. Gene identification and pathway delineation using serves as a potent genetic system, uncovering facts behind evolutionary conservation between flies and humans (1).

Scientific researchers have conducted detailed analyses of human genes, which involved cloning and overexpression. Using the GAL4-UAS system, they were able to study approximately 236 human genes in transgenic flies, representing approximately 1% of the human genome. Through the use of various GAL4 drivers, about 51 transgenes were found to induce reproducible phenotypes when individually expressed in S2 cells. By studying the overexpression of human genes in D. melanogaster, valuable evidence is obtained to support systematic, genome-wide screening and provide a better understanding of gene function in humans (2-5).

Key point

Methodology for cloning genes using *UbiA* as a mammalian cloned gene into the pUAST vector of *Drosophila* as an expression vector.

Range of enzymatic activities of the protein product resulting from the "*heix*1" gene, participating to the synthesis of menaquinone-4 in humans. "*heix*1" gene In *S2*, encodes a protein exhibit sequence homology with the human *UbiA* protein. Progression of various cancers are linked to the loss-of-function mutations present in *UbiA*, including lymphoma, prostate, bladder, carcinoma (6).

Notable mitochondrial defects, abnormal

differentiation, accompanied by hemocyte over-proliferation, manifested by mutations in the "*heix1*" gene (7,8). These results prove that function is conserved of "*heix*1" in human and Drosophila gene, underscore its important role in all cellular processes (9).

Using UAS-GAL4 system in *Drosophila* contribute establishment of stable and selectable plate form (10). Resistance gene in pUAST vector Integration of the hygromycin, coupled with the co-transfection of various pUAST, facilitates the expression of up to four different proteins under hygromycin selection (11). Visual examination of co-transfected cells expressing GFP to a homogeneous cell expressing markers (12).

Objectives

The ultimate goal is to contribute to the ongoing challenge of understanding the diverse biological activities encoded within the human genome. The study suggests that this method can streamline the process of functional genomics research, in investigating the roles of genes.

Materials and Methods

PCR amplification of UbiA gene

Reverse transcription polymerase chain reaction (RT-PCR) was conducted using a cDNA Synthesis Kit (Fermentase®, USA) following the manufacturer's protocol to generate cDNA from extracted RNA. The *UbiA* gene was amplified using specific primers with the following sequences: F primer: CGGAATTCATGGCGGCCT CTCAGGTCCT (introducing an Xbal recognition site, underlined) and R primer: CCGAGCTCTTACTTGTACA GCTCGTCCATGCCG (introducing an XhoI recognition site, underlined) (13). Conditions of PCR included an initial denaturation at 94 °C for 7 minutes, followed by 35 cycles of denaturation at 94 °C for 1 minute, annealing at 62°C for 45 seconds, extension at 72 °C for 1 minutes, and a final extension at 72 °C for 5 minutes using a BioRad C1000 thermal cycler (Marshal Scientific, USA). The PCR product was then analyzed by electrophoresis on a 0.7% agarose gel.

Construction of Recombinant Vector (pcDNA3-UbiA)

The PCR product was purified from agarose gel 1.5% using the AccuPrep® Gel Purification Kit (Bioneer, South Korea) following the manufacturer's instructions. The purified product was subsequently ligated into the pcDNA3 plasmid vector following the manufacturer's protocol. The cloned plasmid was then transformed into Top10 strain bacteria and incubated in Luria-Bertani (LB) broth medium without antibiotics at 37 °C for 1 hour. Afterward, the transformed bacteria were plated on LB agar containing ampicillin (100 mg/mL) and incubated overnight at 37 °C. To confirm gene cloning, the color method was employed. White colonies (indicating successful ligation) and blue colonies (indicating non-recombinant) were identified using the colony-PCR method, enzyme digestion, and sequencing.

Plasmid of white colonies was performed using the Accu-Prep Plasmid MiniPrep DNA Extraction Kit (Bioneer®, South Korea) and digested with Xbal and XhoI enzymes. The enzymatic reactions, conducted under digestion conditions, were incubated for 16 hours at 3 7°C. The resulting products were analyzed by electrophoresis on a 0.7% agarose gel, and their sizes were compared with a 1kb DNA ladder (GeneRuler™ 1kb, Fermentas®, USA). For further verification, the recombinant plasmid (pcDNA3) was sequenced by Bioneer Corporation, South Korea, and the obtained sequence was compared with registered records in the gene bank.

Construction of expression recombinant vector (pcDNA)

pcDNA3 (Invitrogen, Carlsbad, CA, USA) and recombinant plasmid were digested with Xbal and XhoI enzymes. The digested pcDNA3 plasmid and *UbiA* fragment were purified by gel using the DNA Extraction Kit (AccuPrep® Gel Purification Kit, Bioneer, South Korea) (14). The ligation was performed by combining the *UbiA* fragment with the digested pcDNA3 plasmid. The resulting product was then transformed into the Top10 strain following the standard protocol. The transformed bacteria were plated on LB agar plates containing ampicillin (100 mg/mL), and incubated at 37 °C with shaking for 16-18 hours. To confirm the presence of the recombinant pcDNA plasmid, colony-PCR amplification and restriction digestion with Xbal and XhoI enzymes were conducted. The recombinant pcDNA plasmids were further validated by sequencing.

Bacterial growth

To produce competent bacteria, we prepared Difco LB agar by dissolving 40 g in 1 L of distilled water, without antibiotics. We then poured the agar into plates and allowed it to solidify. Subsequently, we grew our "empty" bacteria on these plates at 37 °C for 12 hours.

Next, Difco LB broth media was prepared by dissolving 25 g in 1 L of distilled water, followed by autoclaving the solution at 121°C for 15 minutes. After autoclaving, the sterile LB broth was distributed into flasks, with each flask containing 200 mL of the medium. These flasks were then incubated at room temperature, with sampling commencing 3 hours later. Samples were taken at regular intervals to measure the optical density at 600 nm. The culture was halted when the optical density reached 0.4, indicating the cells had reached a competent state. The cell suspensions were centrifuged at 3000 rpm for 20 minutes at 4 °C, and the pellets obtained were resuspended in 200 mL of transformation buffer. These suspensions were then incubated on ice for 20 minutes. Following this, the samples underwent another round of centrifugation at 3000 rpm for 20 minutes at 4 °C. Finally, the pellets were resuspended in 12 mL of transformation buffer containing 8% DMSO. The cells were then immediately frozen at -80 °C.

Cell culture

PcDNA3.1 was supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/mL), and streptomycin (100 µg/mL) and cultured at 25 °C. S2 cells were cultured in Dulbecco's modified Eagle's medium (Hyclone) supplemented with 10% FBS. TERE-EGFP PcDNA3.1 was cultured in minimum essential medium (MEM, Hyclone) supplemented with 10% FBS. All cultures were maintained at 37 °C in a humidified incubator with 5% CO2 (15).

Inserting the plasmids into the bacteria

The competent bacteria were thawed but not warmed, and they were kept on ice during the procedure (4 °C). The bacteria were split into samples of 100 µL, and 0.1 µg of plasmid was added to each sample (4 °C). The bacteria were kept on ice (4 °C) for 40 minutes, then the samples were put at 42 °C for 30 seconds to transfer the plasmids into the cells ("heat shock"). The samples were kept on ice (4 °C) for two minutes, then each sample was transferred to 1 mL of liquid LB media for 20 minutes at 37 °C on a 300 rpm shaker. The bacteria were distributed on plates containing LB agar with antibiotics and the plates were incubated at 37 °C for 12 hours. The colonies were taken and grown in liquid LB media with antibiotics. (16).

Cloning of the UbiA Gene

Standard molecular biology techniques were used, to clone the *UbiA* into the pUAST vector. The recombinant plasmid was confirmed through sequencing. Enhanced green fluorescent protein (EGFP) vectors pEGFP-N1, pEGFP-C1, (*UbiA*-GFP), mammalian expression vector pcDNA3.1, and TAGBFP were obtained from Invitrogen. Full-length human *UbiA* cDNA was amplified by PCR and cloned into pcDNA3.1. The plasmid was constructed by fusing the N-terminus of *UbiA* and inserting it into pUAST. Then full-length human *UbiA* and pcDNA fragment were cloned separately into the pEGFP-N1 vector to create *UbiA*-EGFP and Apo E-EGFP. *UbiA* was cloned into pUAST to generate pUAST- *UbiA*-EGFP and *UbiA*-BFP.

Plasmid extraction

Plasmid constructions pUASTg was digested with XhoI and XbaI, releasing the loxP site fragment, which was replaced by a duplex oligonucleotide containing the SwaI site (Thermo Fisher). First, we amplified the UAS-hsp70 promoter fragment from pUAST (17). Then, we replaced the SV40 trailer (which was cleaved with KpnI-BamHI) with a duplex oligonucleotide that contains the EcoRI, XhoI, and a BamHI compatible overhang (which will destroy the BamHI site after insertion). After that, we digested the plasmid with NheI-XhoI and inserted a duplex oligonucleotide that contains the *UbiA*-EGFP.

Co-transfection and selection

S2 cells were co-transfected with the cloned UbiA gene and other PUAST constructs using a transfection reagent. To establish stable cell lines, the transfected cells were selected with hygromycin at a concentration of 0.002 µg/mL. The UAS-GAL4 system was employed to drive the expression of the cloned gene and other constructs (Figure 1), utilizing a ubiquitous UAS-GAL4 driver to regulate protein expression.

Transfected cell cultures were maintained with the addition of hygromycin to ensure stable expression. For visual comparison and imaging, cells were also co-transfected with green fluorescent protein (GFP), and the simultaneous expression of GFP was analyzed.

Statistical analysis

GraphPad Prism, ImageJ and Corel Draw software packages were used to analyzed Data.

Results

Gene functions exploration in S2 cell was done by Overexpression screens, although this method lacks appropriate systematic fly strain collections and perfect methods for generating such collections. Loss-of function approaches in *Drosophila* relied on genetic analysis to determine gene's function. Understanding

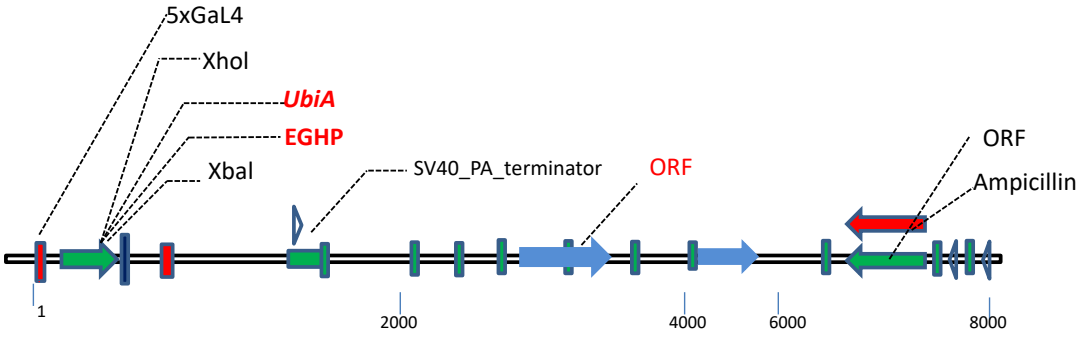

Figure 1. Schematic representation of the multifunctional gene transposon system and insertion screen. The pUAST vector consists of a *UbiA* gene. The protein trap unit is close to the C terminal repeat sequences. In the reverse orientation, close to the C terminal repeat sequences, the enhancer trap reporter GFP (green) is downstream of a short glial fibrillary acidic protein (GFAP) promoter. a polylinker containing unique restriction sites for, XhoI, and XbaI and the SV40 small T intron and polyadenylation site. These features are included in a P element ends (P3′ and P5′) and the white gene which acts as a marker for successful incorporation into the S2 cell genome.

of gene functions have dramatically expanded based on mutagenesis screens (16).

To express human genes in S2 cell, cDNAs were unidirectionally cloned into the P-element transformation vectors, pUAST. These vectors contain the two P-element terminal repeat sequences for mediating germline integration, and a UAS promoter for driving GAL4-dependent gene expression of the cloned gene sequences. Linearization of the pUAST vector for cloning was achieved using the restriction enzymes XbaI and XhoI (Figure 2). However, due to the limited number of suitable cloning sites in this vector, the ability to clone full-length cDNAs is restricted as both restriction enzymes frequently cut in the human genome.

A new vector was developed to improve the efficiency of cloning full-length cDNAs. This vector simplifies the process of transferring full-length inserts from the human cDNA library made in pcDNA3 vectors. By utilizing specific restriction sites for cloning, this new vector enhances the efficiency of full-length cDNA cloning. The pcDNA3 vector greatly improves the cloning efficiency and retrieval of full-length human cDNAs, which is essential for generating transgenic flies (Figure 3).

In the current study, *UbiA* gene sequencing showed 99% identification to the registered sequence in Gen-Bank it was successfully cloned into pUAST vector and into pUAST expression vector for protein (Figure 4). The product of *UbiA* has multiple enzymatic activities, including menaquinone-4 synthesis in human. Peripheral blood mononuclear cells activation of and potentially activates immune functions, MK-4 positively influences the *UbiA* prenyltransferase domain-containing 1 (*UbiA*), to produce MK-4. *UbiA* expression and the effects of MKs have not yet been studied in mammalian endometrial epithelial cells (Figure 5). To ensure success of cloning, western blot for whole protein in control and transfected S2 cells was conducted (Figure 6).

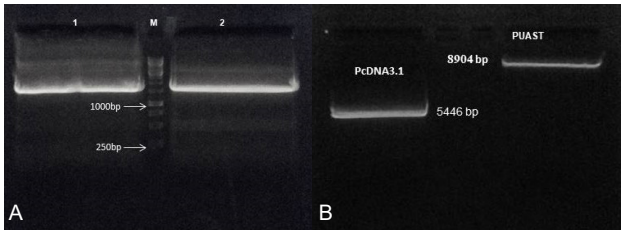

Figure 2. (**A**) Gel electrophoresis techniques for separating DNA molecules of pUAST vector. (**B**) Two fragments of digestion pUAST vector and pcDNA 3 containing restriction enzymes Xba and XhoI.

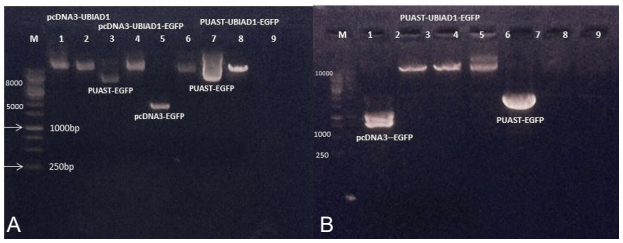

Figure 3. (A) Gel electrophoresis results for separating DNA molecules: Lane 1. E pUAST vector; Lane 3. UbiA-PcDNA3.1 mammalian vector; Lane 4. pUAST-EGFP fly vector; Lane 6, 8. pUAST-UbiA-EGFP; Lane 7. pUAST-EGFP. (B) Gel electrophoresis results: Lane 1. Contains two fragments from digestion by EcoRI and XhoI restriction enzymes, Top10 and PcDNA3-EGFP; Lanes 2, 3, 4, 5. pUAST-UbiA-EGFP.

Discussion

Our study focused on characterizing a REC-based method for cloning human genes into the pUAST vector. This method was designed to be secure, swift, and straightforward. We utilized P-element transformation vectors, specifically pUAST (18), as the basis of our strategy.

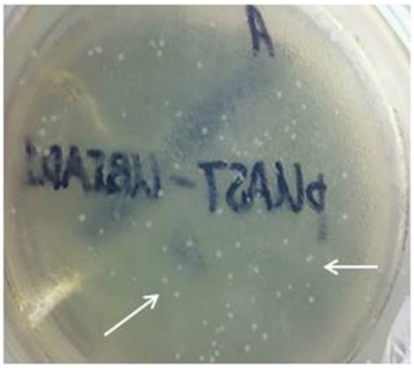

Figure 4. Detection of *UbiA* gene of EGFP-positive clones after mutagenesis. Distribute your bacteria's in plates containing LB agar with antibiotics. Incubate the plates at 37 °C for 12 h. Take the colonies and grow up then in liquid LB media with antibiotics.

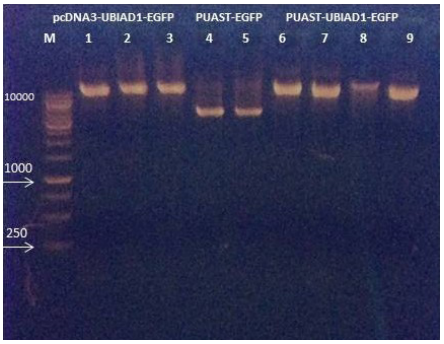

Figure 5. Gel electrophoresis techniques for separating DNA molecules. PcDNA3-*UbiA*-EGFP mammalians vector lane 1-3. pUAST-EGFP vector lane 4, 5. pUAST-*UbiA*-EGFB gene and vector lane 6-9.

Figure 6. Western blot analysis of protein extracts from encoding for wild-type (Control) and *heix1* mutant gene of transfected S2 cells.

The pUAST vector contains essential elements such as the mini-white+ gene as a visible marker, two P-element terminal repeat sequences for germ line integration, and a UAS promoter for driving GAL4-dependent gene expression.

pUAST vector was linearized using the restriction enzymes XbaI and XhoI to begin the cloning process. However, a frequent cutting sites limitation arose from these enzymes in human genome, minimizing the number of full-length cloned *UbiA* cDNAs (Figure 3). To treat this obstacle, pUAST vector was modified, improving the efficiency of cloning *UbiA* cDNAs. This process allowed for full-length inserts from the human *UbiA* cDNA library by specific restriction sites.

The GAL4-UAS ectopic expression system was employed, which is a valuable tool for transgene overexpression in *D. melanogaster* (18). The UAS promoter activates transgene expression through GAL4 under various promoters, ensuring that there is no interference with the natural promoters (19).

We selected distinct GAL4 drivers from the Flybase database for high-throughput analysis, (20). These drivers have multiple expression patterns, from ubiquitous to compartment-specific or cell type-specific, enabling high-throughput analysis.

Crossing these selected GAL4 drivers with flies carrying human transgenes resulted in diverse phenotypes. Interestingly, the S2 cell gene homologs of the same human genes induced similar apoptotic phenotypes upon overexpression, indicating conserved functional relevance across species (2, 21).

In the field of molecular biology, various cloning technologies have been developed as substitutes for restriction enzyme-based cloning (REC). Nonetheless, despite the advancement of these methods, REC continues to be crucial for constructing recombinant plasmids, primarily because newer techniques present certain challenges. These challenges encompass technical complexities, multiple cloning steps, the requirement for long primers, and difficulties in acquiring molecular biology materials, especially in developing nations (22, 23).

The loss of heix1 expression in D.M may be a defect in the signaling process between mitochondria and the nucleus that tumors utilize to separate vitamin K-mediated oxidative stress signaling from apoptosis or negative growth signaling through cholesterol elevation. This study successfully replaced the *heix* gene with *UbiA*, enabling researchers to exchange compatible genes between humans and *Drosophila*. This breakthrough opens doors for improved investigations and a better understanding of the function of human genes (24-25).

Conclusion

In conclusion, our study emphasizes the ongoing importance of REC in recombinant plasmid construction, particularly when cloning human genes into the pUAST vector. By combining REC with the GAL4-UAS system and a variety of GAL4 drivers, we have established a robust platform for investigating the functional roles of human genes in *Drosophila*. This approach provides valuable insights into the shared developmental processes between humans and flies.

Limitations of the study

The study faced a challenge in cloning a human gene in pUAST plasmid regarding differences in volume of the gene (base pair).

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Authors' contribution

Conceptualization: Zainab Sabeeh Al-Allak.
Data curation: Zainab Zamil Gataa Allami.
Formal analysis: Maytham Abdulkahdim Dragh.
Funding acquisition: own funding.
Investigation: Maytham Abdulkahdim Dragh.
Methodology: Maytham Abdulkahdim Dragh.
Project administration: Zainab Sabeeh Al-Allak.
Resources: Zainab Zamil Gataa Allami.
Software: Maytham Abdulkahdim Dragh.
Supervision: Maytham Abdulkahdim Dragh.
Validation: Zainab Sabeeh Al-Allak.
Visualization: Zainab Zamil Gataa Allami.
Writing–original draft: Zainab Sabeeh Al-Allak.
Writing–review & editing: Zainab Sabeeh Al-Allak.

Conflicts of interest

The authors declare that they have no competing interests.

Ethical issues

The research followed the tenets of the Declaration of Helsinki. The Ethics Committee of University of Misan college of science in 2023 approved this study (Ethical code #Dept362). Accordingly, written informed consent was taken from all participants before the study. Moreover, ethical issues (including plagiarism, data fabrication, and double publication) were completely observed by the authors.

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