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CLONING AND CHARACTERIZATION OF COMPLEMENTARY DNA ENCODING THE EUKARYOTIC INITIATION FACTOR-2 ASSOCIATED 67 kDa POLYPEPTIDE (p^{67})

by

Shiyong Wu

A DISSERTATION

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DISSERTATION TITLE

Cloning and Characterization of Complementary DNA Encoding the Eukaryotic Initiation Factor 2 (eIF-2) Associated 67 kDa Protein (p67)

BY

SHIYONG WU

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GRADUATE COLLEGE
UNIVERSITY OF NEBRASKA

Cloning and Characterization of Complementary DNA Encoding The Eukaryotic Initiation Factor-2 Associated 67 kDa Polypeptide (p^{67})

Shiyong Wu, Ph.D.

University of Nebraska - Lincoln, 1992

Adviser: Naba K. Gupta

An eukaryotic initiation factor-2 (eIF-2) associated 67 kDa polypeptide (p^{67}) has been known to play a critical role in the regulation of protein synthesis initiation in mammalian cells. Under certain physiological conditions, the eIF-2 α-subunit can be phosphorylated by several protein kinases, leading to eIF-2 inactivation and hence protein synthesis inhibition. As p^{67} can protect the eIF-2 α-subunit from phosphorylation, it promotes protein synthesis in the presence of active eIF-2 kinases.

In order to further study the activities of p^{67}, we cloned the p^{67} gene using the following procedures:

1. A cDNA library in λgt 11 was constructed from a cultured rat hepatoma (KRC-7) cell line which is highly enriched in p^{67}.
2. P^{67} was cleaved using chemical and enzymatic methods. Stretches of amino acid sequence were obtained from p^{67} using Edman degradation.
3. Oligonucleotides that corresponded to the peptides sequence of p^{67} were synthesized and used to amplify the gene encoding p^{67} with the polymerase chain

reaction (PCR). Southern blot analysis and DNA sequencing proved that one of the PCR products was part of the p^{67} gene.

4. The PCR product was ^{32}P labeled and used as a probe to screen the λgt 11 library. One p^{67} gene, which contained approximately 2 kilobases (kb), was cloned and characterized.

5. The gene was subcloned into pGEM5zf(+) vector and sequenced by the Sanger method. It was found that: (1) The gene contains a total of 1953 base-pairs (bp). (2) It has a 1440 bp open reading frame that encodes a protein with a calculated molecular weight of 53190.78 kDa. (3) The amino acid sequence deduced from the DNA sequence matches the sequence of the p^{67} peptide fragments. (4) It contains an strong initiation sequence (AACATGG). (5) It has a poly(A) additional signal (AATAAA) 14 bases upstream of a $poly(A)_9$ tail.

6. The subcloned p^{67} gene was translated *in vitro*. The product had a molecular weight of 65 kDa on SDS-PAGE and was immunoprecipitated by anti-p^{67} polyclonal antibodies. This 65 kDa polypeptide interacted *in vitro* with eIF-2.

PREFACE

The format of this dissertation represents a departure from the conventional thesis style. The thesis has been divided into two sections. Section I is the general introduction and short review of the published literature in this area of research. Section II represents a complete manuscript.

The results present in Section II has been submitted for publication.

Reference:

1. Wu, S., Gupta, S., Chatterjee, N., Hileman, R. E., Chakrabarti, D., Danslow, N. D., Merrick, W. C., Kinzy, T. G., Osterman, J. C. and Gupta, N. K. (1992) "Cloning and Characterization of Complementary DNA Encoding the Eukaryotic Initiation Factor 2 (eIF-2) Associated 67 kDa Protein (p^{67})", submitted for publication.

2. Wu, S., Gupta. S., Ray, M., Hileman, R. E., Chakraborty, D, Denslow, N. D., Osterman, J. C. and Gupta, N. K. (1992) "Peptide chain initiation factor, p^{67}: Characteristics, gene cloning and possible therapeutic uses." In "Down Stream Processing In Biotechnology" (R. N. Mukherjee, Ed.) TATA McGnaw Hill, Wiley Eastern Limited (India) (In Press).

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Dedicated to

my wife, **Limin Xi**

my parents

Long-ge Wu and **Ding-ding Wu**

and

my son, **Larry S. Wu**

ABBREVIATIONS

AMV	avian myeloblastosis vires
bp	base-pair
BSA	bovine serum albumin
CIP	calf intestinr phosphatase
DEPC	diethyl pyrocarbonate
DMEM	Dulbecco's modified Eagle's medium
dsI	double-stranded RNA activated inhibitor
EDTA	ethylenediaminetetreacetic acid
eIF-2	eukaryotic initiation factor-2
EtBr	ethidium bromide
Exo III	exonuclease III
FPLC	fast protein liquid chromatograph
GEF	guainine nucleotide exchange factor
GlcNAc	N-acetyl glucosamine
HRI	heme-regulated inhibitor
IPTG	isopropyl-β-D-thiogalactoside
kb	kilobase
LB	Luria Bertoni
NT-40	nonidet P-40
p^{67}	eIF-2 associated 67 kDa polypeptide
PAGE	polyacrylamide gel electrophoresis

PEG	polyethylene glyco
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pfu	plaque forming units
PMA	phorbol-12-myristate-13-acetate
PNK	polynucleotide kinase
SAAP	streptavidin-alkaline phosphatase
SDS	sodium dodecyl sulfate
TAE	Tris acetat EDTA
TE	Tris EDTA
Tris	N-tris (hydroxymethyl) aminomethane
vol	volume
wt	weight
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

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Section I

The Roles of eIF-2 Associated 67 kDa Peptide (p^{67}) In Regulation of Protein Synthesis Initiation, And Gene Cloning of p^{67}

The regulation of gene expression in eukaryotic cells is one of the most actively studied areas in molecular biology. The genetic information encoded in DNA flows to messenger RNA (transcription) and finally to protein (translation). Gene expression in eukaryotic cells is regulated at the transcriptional level, as well as at the translational level. One well known mechanism for translational regulation is the phosphorylation of the eIF-2 (eukaryotic initiation factor 2). This phosphorylation leads to inactivation of eIF-2, hence inhibition of protein synthesis[1].

Protein synthesis is a complex process which involves a large number of macromolecules, including mRNA, tRNA, enzymes, protein factors and ribosomes. The first step in the initiation of protein synthesis is the formation of a ternary complex with eIF-2 , GTP (guanidine triphosphate) and Met-$tRNA_f$ (methionyl initiator transfer RNA)[1-3]. In the presence of mature mRNA and other protein factors, such as Co-eIF-2A and Co-eIF-2C, the 40S ribosome interacts then with the ternary complex. This results in the formation of a Met-$tRNA_f$·mRNA·40S complex, called the 40S complex[4]. After the anticodon of the Met-$tRNA_f$ pairs with the initiation codon on the mRNA, the 40S complex binds to the 60S ribosomal subunit to form an 80S complex: the Met-$tRNA_f$·mRNA·80S complex. The 80S complex participates in polypeptide chain elongation[4]. In Dr. Gupta's laboratory, we study the mechanism and regulation of protein synthesis initiation.

The key factor in protein synthesis initiation is eIF-2. This protein contains three subunits: α (38 kDa), β (54 kDa) and γ (52 kDa). The activity of eIF-2 is regulated by several protein synthesis inhibitors, such as HRI (heme-regulated inhibitor)[5] and dsI

(double-stranded RNA activated inhibitor)[6]. These inhibitors phosphorylate the eIF-2 α-subunit and thereby inactive eIF-2. It was assumed that eIF-2 α(P) forms an abortive complex with GEF (guanine nucleotide exchange factor) and GDP[7]. This eIF-2 α-subunit phosphorylation mechanism is widely used in animal cells to regulate protein synthesis under different physiological conditions, such as viral infection[8-14], heat shock[15,16] and nutritional starvation[17]. Our knowledge about this mechanism of translation regulation is based primarily on the studies of protein synthesis in reticulocyte lysate. In rabbit reticulocyte lysates, protein synthesis is dependent on the presence of heme. In heme - deficient lysates, protein synthesis starts at a normal rate but declines within a few minutes. It is generally believed that a protein kinase - HRI, becomes activated in the absence of hemin. The activated kinase then phosphorylates the α-subunit of eIF-2 and thus protein synthesis is inhibited. For over a decade, almost all the laboratories doing research in this field reported that addition of exogenous eIF-2 to the heme-deficient reticulocyte lysates could relieve the translational inhibition and hence promote protein synthesis[18-21]. In 1978, Dr. Gupta's laboratory reported that a homogeneous eIF-2 only composed of three subunits (α, β and γ) could efficiently form the ternary complex and the 40s complex, but could not reverse protein synthesis inhibition in heme-deficient reticulocyte lysate. The laboratory found that only a partially purified eIF-2 reversed protein synthesis inhibition in heme-deficient reticulocyte lysate[22]. In 1988, dr. Gupta's laboratory reported that a partially purified eIF-2 containing the α, β and γ subunits and an extra 67 kDa peptide (p^{67}) reversed the protein synthesis inhibition in heme-deficient reticulocyte lysates[23]. The 67 kDa peptide protects the eIF-2 α-subunit from eIF-2 kinase

catalyzed phosphorylation and thus promotes protein synthesis in the presence of active kinase(s). In addition, p^{67} binds specifically to the eIF-2 γ-subunit. This binding to the γ subunit is necessary for p^{67} to protect the eIF-2 α-subunit from phosphorylation. P^{67} is a glycoprotein and contains multiple O-linked GlcNAc (N-acetyl-glucosamin) residues. These glycosyl residues are necessary for p^{67} activity, but not necessary for p^{67} binding to eIF-2[24].

Recently, a series of experiments were done in Dr. Gupta's laboratory to study the roles of p^{67} in the regulation of protein synthesis initiation in different cell lysates and a cultured animal cell line - KRC-7 (derived from Reuber H35 rat hepatoma cells)[25]. The experimental results indicated that: (1) Under normal growth conditions, eIF-2 kinase(s) are present in active forms in all animal cells. The eIF-2 α-subunit is protected from phosphorylation because of the activity of p^{67}. P^{67} protects the eIF-2 α-subunit from phosphorylation, thereby promoting protein synthesis in the presence of active eIF-2 kinase(s). (2) In both heme-supplemented and heme-deficient reticulocyte lysates, HRI is always in the active form. However, in heme-deficient reticulocyte lysate, p^{67} is deglycosylated and subsequently degraded. This result suggests that protein synthesis inhibition in heme-deficient reticulocyte lysate is not due to activation of HRI, but rather due to the degradation of p^{67}. (3) In serum starved KRC-7 cells, p^{67} is also degraded. Addition of a mitogen PMA (phorbol-12-myristate-13-acetate) to starved cells induces the p^{67} level and hence increases protein synthesis activity. The results show that p^{67} is both inducible and degradable. The study demonstrates that the p^{67} level correlates directly to the protein synthesis activity of the cell, indicating that p^{67} is a critical factor in protein

synthesis regulation in animal cells.

Since p^{67} plays such an important role in the regulation of protein synthesis, it becomes essential to clone the gene encoding p^{67}. Modern molecular biology techniques have made such a cloning project possible. The availability of reverse transcriptase, an RNA dependent DNA polymerase, made it possible to synthesize cDNA (complementary DNA) using mRNA as a template. Compared with genomic DNA, cDNA has several advantages in eukaryotic gene cloning. The cDNA comes from mRNA and in many cases, the level of mRNA in the cells is inducible. Therefore, the gene of interest sequence can be enriched in a cDNA library. As p^{67} level is very high in confluent KRC-7 cells and its level is further increased by PMA stimulation[25], we constructed our cDNA library in λgt 11 using mRNA from confluent KRC-7 cells after two hours of PMA addition. cDNA contains much less non-coding sequence than genomic DNA as it has no introns. As a result, the cDNA sequence can be directly translated into protein.

The cDNA was packaged into λgt 11 bacteriophage[26,27]. The advantages of using λgt 11 vector are a high cloning efficiency, easy of handling and maintenance, and straight forward screening by either radioactively labeled oligonucleotides or antibodies. In our laboratory, the KRC-7 cDNA library in λgt 11 was screened using a ^{32}P labeled p^{67} cDNA fragment which was a PCR (polymerase chain reaction)[28,29] product.

PCR, the reaction of the last decade, is a powerful tool in molecular biology. It amplifies the amount of a specific DNA sequence in a manner of 2^n by thermocycles - melting, annealing and elongation. PCR means that a single copy gene can be amplified to $1x10^9$ copies of gene after 30 cycles. The p^{67} cDNA fragment was amplified using two