Identification of Protein-RNA and Protein-Protein Interactions by the Neuronal HuC Protein of *Mus musculus*

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Statement:

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Abstract:

Post-transcriptional gene regulation is an essential process by which all vertebrate organisms regulate gene expression across a wide array of cell types. Such regulation is of particular importance within neurons, as the highly polarised nature of such cells requires that gene expression be tightly regulated in a spatio-temporal manner throughout both proximal and distal regions of the cell. RNA-binding proteins have been shown to regulate a wide range of post-transcriptional regulatory processes, and one family of vertebrate RNA-binding proteins, the Hu family, has been implicated in neuronal specification and differentiation. Of the four proteins in the Hu family, two (HuC and HuD) are uniquely neuronal, whilst one (HuB) is expressed both in neurons and the early embryo. The fourth (HuA) is expressed ubiquitously. Each of the four Hu proteins binds RNA in a sequence-specific manner, mediated by three RNA recognition motifs (RRMs) present in each family member, and are believed to regulate mRNA translation, localisation, and/or message stability. However, little information is currently available regarding the molecular mechanisms by which the neuronal Hu proteins mediate their effects. Thus, whilst HuA has been demonstrated to bind to AU-rich elements (AREs) in the 3'UTRs of a number of labile mRNA messages, little information is currently available regarding the specific RNA sequences that mediate RNA binding by the neuronal Hu proteins. Furthermore, a number of studies have demonstrated that the function of HuA relies upon a number of direct protein-protein interactions. However, to date few interactions have been identified for the neuronal Hu proteins. Identification of the RNA sequences bound by the neuronal Hu proteins and examination of the protein cofactors that mediate the function of these proteins in respect to such bound messages is essential in order to develop an understanding, both of the role played by the neuronal Hu proteins during development, and of the mechanisms by which these functions are achieved.

Herein we describe the development of a protocol for the efficient expression and purification of active recombinant HuC, in addition to the subsequent application of this protein to an investigation of the mRNA sequence motifs that mediate RNA binding by this neuronal Hu family member. Moreover, we demonstrate that HuC preferentially binds to RNAs containing short, interspersed uridine-rich sequence motifs *in vitro*. Additionally, we present evidence which suggests that Hu-bound RNA sequences identified by the Cross-Linking and Immunoprecipitation (CLIP) method do not necessarily represent sites of direct HuC binding, and thus that further study is required in order to identify the complement of mRNAs bound by each neuronal Hu protein. Furthermore, we describe the identification of a number of putative HuC protein-protein interactors by means of yeast two-hybrid analysis, in addition to the development of a protocol for the specific immunoprecipitation of the neuronal Hu proteins. This protocol may be applied both to the confirmation of putative protein interaction partners, and the identification of further protein interactors that mediate the function of the neuronal Hu proteins during neuronal development. Together, these studies represent the necessary first steps toward a complete understanding of the molecular mechanisms that underlie the function of these proteins, and provide an essential foundation for further studies on the role of the neuronal Hu family proteins during neuronal development.

Abbreviations:

AbA	Aureobasidin A
APS	ammonium persulfate
ARE	AU-rich element
ATP	adenosine triphosphate
bp	base pair(s)
ccpm	Cerenkov counts per minute
cDNA	complementary deoxyribonucleic acid
CIP	calf intestinal phosphatise
CLIP	cross-linking and immunoprecipitation
CNS	central nervous system
CPI	complete protease inhibitor
cpm	counts per minute
Cy3	cyanine3
Cy5	cyanine5
DAPI	4',6-damidino-2-phenylindole
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylene diamine tetraacetic acid
FITC	fluorescein isothiocyanate
GFP	green fluorescent protein
GST	glutathione-S-transferase
HEPES	4-2-hydroxyethyl-1-piperazineethanesulfonic acid
His_n	n-histidine polypeptide
hpf	hours post-fertilisation
HRP	horseradish peroxidise

IEF	isoelectric focusing
IgG	immunoglobulin G
kb	kilobase(s)
K_d	dissociation constant
kDa	kiloDalton(s)
mHuCsv1	murine HuC Splice Variant 1
min	minute(s)
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	messenger ribonucleic acid
NHS	normal human serum
nHu	neuronal Hu protein(s)
Ni-IDA	nickel-iminodiacetic acid
NP-40	nonidet P40
nt	nucleotide(s)
NTP	nucleotide triphosphate
OD_{600}	optical density at 600 nm
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBST	phosphate buffered saline with 0.1% Tween-20
PCR	polymerase chain reaction
pI	isoelectric point
PND	paraneoplastic neurological disorder
PNK	polynucleotide kinase
PNS	peripheral nervous system
PVDF	polyvinylidene fluoride
RNA	ribonucleic acid
RNP	ribonucleoprotein
RRM	RNA recognition motif

SDS	sodium dodecyl sulphate
sv	splice variant
TBE	tris-borate EDTA buffer
TEMED	tetramethylethylenediamine
TEV	TEV protease site
UTR	untranslated region
UV	ultraviolet
V	volt(s)

Standard one-letter abbreviations for nucleotides and three-letter abbreviations for amino acids are also used.