생명과학회지
Korean J. Life Science, 7(3), 167−171, 1997

Isolation of cDNA Encoding Double-Stranded RNA Binding Protein (RBFII)

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Abstract

As an initial effort to elucidate RNA : protein binding in a way to regulate translation initiation and phosphorylation, a cDNA encoding a double-stranded RNA binding factor (RBFII) was isolated from HeLa ZAPII cDNA library by affinity screening using [$\alpha-^{32}P$]UMP-labeled HIV Rev-responsive element (RRE) RNA. The nucleotide sequence of RBFII cDNA was determined and revealed to contain a common ORF in most part with the previously characterized RBF (or TRBP) cDNA except the 5' end. At the 5' end, This common ORF was fused in-frame to N-terminal residues of Lac-Z through a unique 138 nt sequence encoding 46 residues in the case of RBFII and a 63 nt sequence encoding 21 residues in the case of RBFI. The context of ATG appearing first in the sequences suggests that both these cDNA inserts are incomplete at the 5' end.

Key words : Double-stranded RNA binding factor (RBFII), Affinity screening

Introduction

RNA : protein interactions mediate many fundamental mechanisms during gene expression[1,2], mRNA transcription[3,4], posttranscriptional processing including polyadenylation[5], and translation[6,7]. Many cellular and viral RNA binding proteins that bind unique RNA targets and introduce specificities into the general regulatory mechanisms have been identified[8,9]. In the course of RNA: protein interactions, protein phosphorylation is also concomitant regulatory event critical to gene expression. For example, global regulation of eukaryotic mRNA translation is mediated by protein phosphorylation events, most notably at the translation initiation step, that involve eukaryotic initiation factor 2 (eIF-2). A ribosome-associated protein kinase also called PKR phosphorylates eIF-2α upon activation by double-stranded (ds) RNA[10,11]. PKR is involved in the antiviral and antiproliferative activity and cellular differentiation[12].

In recent reports[13,14,15], a cDNA encoding a HeLa cellular protein isolated on the basis of a selective reactivity to HIV-1 Rev-responsive element RNA has been described to show that the protein complemented the inhibition of protein synthesis upon the localized activation of PKR *in vivo* and inhibited PKR phosphorylation upon activation by double stranded RNA *in vitro*, suggesting its role for mediating the inhibition of PKR activation and stimulating translation in a localized manner. In this manuscript, I describe RBF cDNA (designated RBFII) isolated from HeLa ZAPII cDNA library by affi-

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nity screening using the [$\alpha-{}^{32}P$]UMP-labeled HIV Rev-Responsive Element (RRE) RNA. The nucleotide sequence and deduced amino acid sequence of RBFII is presented in comparison to RBF.

Materials and Methods

RNA probe

DNA template for HIV-1 Rev-responsive element (RRE) RNA transcript[16] in the reaction mixture containing T7 promoter-tagged promer was amplified by polymerase chain reaction for 30 cycles, each cycle constituting 94℃ for 30 sec, 55℃ for 30 sec and 72℃ for 90 sec. Denaturation of template DNA at 95℃ for 5 min was performed prior to the cycle and postreaction at 72℃ was for 5 min. PCR product was electrophoresed in 2% agarose gel and purified by Gene-clean method (Bio101, USA). *In vitro* transcription was by use of a commercial T7 transcription kit (Stratagene, USA). [$\alpha-{}^{32}P$]UMP labelled transcription products were extracted with phenol : chloroform and with chloroform : isoamylalcohol and purified by Sephadex G-50 chromatography. RNA purity was determined by polyacrylamide gel electrophoresis (PAGE) in 8 M urea, and the transcripts were purified from gels as necessary[16]. The gel-purified RNAs were denatured by boiling and self-annealed by slow cooling to 50℃ in 0.2 M NaCl over a 60 min period.

Isolation of RBFII cDNA clone

The ZAPII HeLa cDNA library (Stratagene, USA) was screened using [$\alpha-{}^{32}P$]UMP labelled RNA. Basically, I adapted the screening protocols for the isolation of DNA binding clones (Stratagene). Nitrocellulose filters soaked with 10 mM IPTG were overlaid onto phage plates (5×10^4 plaques per plate) for 3 hr at 37℃. The filters were then immersed in Tris buffer containing 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM DTT supplemented with 5% nonfat dried milk (NFDM) for 1 hr followed with rinsing with the same buffer containing NFDM and brief drying on the Whatmann paper. The filters were then immersed in HEPES buffer containing 25 mM HEPES, pH 7.9, 25 mM NaCl, 5 mM $MgCl_2$, 0.5 mM DTT supplemented with 6M guanidine-HCl (GuHCl) for 20 min. The buffer was gradually diluted to remove GuHCl. The filters were incubated in Tris buffer with NFDM for 30 min, rinsed with HEPES buffer, transfered to Tris buffer, and incubated with the labeled RRE RNA probe (1×10^7 cpm per 10 filters in 50 ml buffer in 155 mm dish) and yeast tRNA (0.2 mg/ml) for 1 hr. The filters were washed with Tris Buffer 3 to 4 times, air-dried, and exposed to X-ray films at −70℃. Thereafter, radioactive plaques were amplified and rescreened three more times.

Nucleotide sequence analysis

Positive lambda ZAPII clones were autoexcised to produce recombinant pBluescript SK- plasmid containing putative RRE RNA binding protein according to Stratagene's protocols. Nucleotide sequence was determined by the dideoxy chain termination method[17]. For complete cDNA sequence determination, oligonucleotides directed for both sense and antisense were designed and used as primers for defining inward sequence contents following each round of DNA sequence analysis.

Results and Discussion

Commercial ZAPII lambda coliphage recombinant cDNA expression library of HeLa cell mRNAs was affinity screened using ^{32}P-UMP labeled RRE RNA. Approximately 2×10^7 recombinant lambda plaques were transferred to nitrocellulose filters after IPTG induction to express the Lac-Z fusion proteins. Two positive plaques demonstrating consistent RRE-binding were selected after three rounds of serial screening (Fig. 1). By the use of a VCS-M 13 inteference resistant helper phage (Materials and Methods), the recombinant cDNA inserts from the lambda

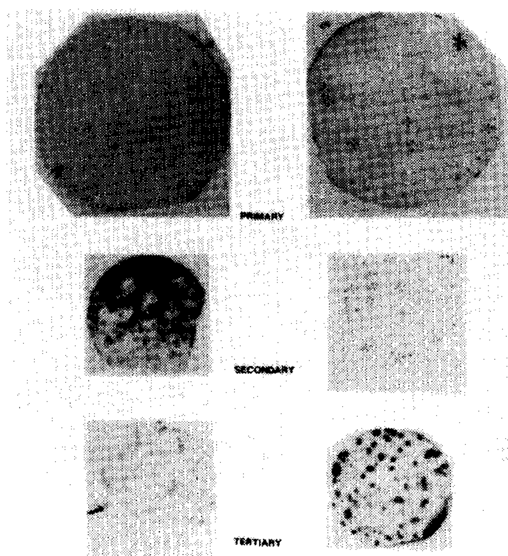

Fig. 1. Recovery of cDNA clones encoding RRE RNA binding cellular proteins.
Top to bottom : primary, secondary and tertiary screening step, respectively.
Left to right : RBFI and RBFII.

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(RBFI)
AAT TCC GCT CTT GGG TTC TGT AGT TTT CTC GCG ATC CAA AAG GCT
 N   S   A   L   G   F   C   S   F   L   A   I   Q   K   A
CCG TGC CCA AAG CAA ATG
 P   C   P   K   Q   M (21)
(RBFII)
AAT TCC GTA GGC TGT GTA TTG GGG CGC GTG GAG GCT GCA GTC ACG
 N   S   V   G   C   V   L   G   R   V   E   A   A   V   T
GTG GCG CCC GCG GGG ACG GAG GAG GGA ATG AGT GAA GAG GAG CAA
 V   A   P   A   G   T   E   E   G   M   S   E   E   E   Q
GGC TCC GGC ACT ACC ACG GGC TGC AGG CTG CCT AGT ATA GAG CAA
 G   S   G   T   T   T   G   C   R   L   P   S   I   E   Q
ATA
 I (46)
(Common region)
LAANPGKTPI SLLQEYGTRI GKTPVYDLLK AEGQAHQPNF TFRVTVGDTS  50
CTGQGPSKKA AKHKAAEVAL KHLKGGSMLE PALEDSSSFS PLDSSLPEDI 100
PVFTAAAAAT PVPSVVLTRS PAMELQPPVS PQQSECNPVG ALQELVVQKG 150
WRLPEYTVTQ ESGPAHRKEF TMTCRVERFI EIGSGTSKKL AKRNAAAKML 200
LRVHTVPLDA RDGNEVEPDD DHFSIGVGFR LDGLRNRGPG CTWDSLRNSV 250
GEKILSLRSC SLGSLGALGP ACCRVLSELS EEQAFHVSYL DIEELSLSGL 300
CQCLVELSTQ PATVCHGSAT TREAARGEAA RRALQYLKIM AGSK       344
tgaagcccca gctggactca tggatgtgca ccctttgctc cctgctcttt
ctgcctctgg gctcatgtat ctgcgcagct ctggtaccct ctgtgggtgc
catctctacc tctgacacag actgcctgcc ttgaagctga gaaggcacag
ggcaaggagc caaggaccac agagcctcag ccagcccagg atccgtcctc
attttattgg tgatgatgaa tgggaatgaa atcagggggc tgtctactag
agcctggaat aaatatgctg ctttgtgga
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Fig. 2. Comparison of nucleotide sequence and deduced amino acid sequence of RBFI and RBFII cDNA.

phage DNA were autoexcised to generate two recombinant pBluescript SK- plasmids (pRBFI and pRBFII) harboring the coding sequence for the putative RRE RNA binding proteins linked to β-galactosidase promoter. RBFI, reported as RBF or TRBP elsewhere[13,14] but, for distinction to RBFII, designated so in this report, was characterized to have a inhibitory effect to double-stranded RNA dependent protein kinase (PKR) and its molecular features *in vivo* and *in vitro* were described elsewhere[13,14,15]. For comparison, pRBFII was analyzed by restriction enzyme digestion and agarose gel electrophoresis to show that its insert cDNA was slightly longer and different in part in restriction mapping (data not shown). RBFII cDNA sequence was completely determined. Its molecular structure was compared to RBFI and the translated sequence of both cDNA inserts is presented in Fig. 2. Both pSK-RBFI and pSK-RBFII had a common DNA sequence of 1032 nt encoding a long ORF of 344 amino acids that was identical to the TAR RNA binding protein, TRBP[18]. Previous work with TRBP had shown significant protein homology between a contiguous stretch of 59 amino acids of RBFI (residues 150 −208) and a similar basic residue rich block in *E. coli* RNAse III, a dsRNA binding protein. When RBFI was analyzed for internally redundant sequence, a long stretch of 58 amino acids between positions 34 to 91 was a homologous to a second stretch of 55 residues between 163 to 218. These two motifs in the case of RBFII reside at residues 55 to 102 and 184 to 239, respectively. At the 5' end, this common ORF was fused in-frame to the 37 N-terminal residues of lac-Z through a unique 63 nt sequence encoding 21 residues in the case of RBFI and 138 nt sequence encoding 46 residues in the case RBFII. This arrangement predicted an aggregate of 427 residues with a calculated molecular mass of 45 kDa for RBFII and 402 residues with a mass of

42 kDa for RBFI. Neither RBFI nor RBFII had a MET start codon suggesting that both these cDNA inserts were incomplete at the 5' end. Alternate translation initiation loci may be suggested, but the general context including MET start codon (PuPyPyPuPyPyATGG)[19] excludes a possibility that the first appearing ATG from each sequence (GAGGGAATGA for RBFII, AAGCAAATGC for RBFI) is recognized as a MET start codon. Therefore, an mRNA structure with a common MET start codon upstream under differential splicing process may be another possibility ; however, analysis of full-length RBF cDNA structure is required.

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초록 : 이중선RNA결합단백질(RBFII)의 cDNA분리

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번역개시 및 인산화의 조절에 관여하는 RNA와 단백질의 결합 및 인식기작을 연구하기 위해서 [$\alpha-^{32}P$] UMP-labeled HIV Rev-responsive element (RRE) RNA를 이용한 affinity screening에 의해서 HeLa ZA-PII cDNA library로 부터 이중선RNA결합단백질의 cDNA (RBFII)를 분리하였다. RBFII의 cDNA에 대한 염기서열을 결정하였으며 기존에 연구된 바 있는 RBFI (RBF 또는 TRBP로 보고되었으며 본 연구에서는 RBFII와 구분하기 위해 RBFI으로 명명)과 대부분의 경우 공통적인 ORF를 지니는 것으로 나타났다. 그러나 5' 말단에서는 공통적인 ORF가 RBFI의 경우 21개의 아미노산을 의미하는 63 nt가 Lac-Z의 N-말단에 연결된데 비해서 특이한 46개 아미노기륵 의미하는 138 nt가 존재함이 밝혀졌다. 5'-말단에 처음 나타나는 ATG 및 부근의 염기서열을 분석해 볼 때 양 cDNA는 5' 말단이 완전하지 않은 것으로 사료된다.