International Journal of Biological Macromolecules 45 (2009) 157–162

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Involvement of PU.1 in mouse *adar-1* gene transcription induced by high-dose esiRNA☆

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ARTICLE INFO

Article history:
Received 17 March 2009
Received in revised form 24 April 2009
Accepted 24 April 2009
Available online 5 May 2009

Keywords:
RNA interference
Adar-1
PU.1
RNAi negative regulation

ABSTRACT

Adar-1 gene plays an important role in the negative regulation of RNA interference. We previously showed that increased *adar-1* mRNA level was associated with the rebound of gene expression after RNAi suppression. In this study, we identified a PU.1 binding site upstream from transcription start point of *adar-1* gene and is essential for the promoter activity. Knockdown and over-expression of the PU.1 gene resulted in decreased and increased activity of *adar-1* promoter, respectively. Our results suggest that transcription factor PU.1, could bind to the *adar-1* promoter and play a key role in activating transcription of gene induced by high-dose esiRNAs.

1. Introduction

RNA interference (RNAi), selective degradation of cognate mRNAs by interference RNA (siRNA), is a post-transcriptional gene silencing mechanism conserved among eukaryotic organisms [1]. RNAi is involved in several biological processes, including anti-virus reaction, inhibition of transposition and gene expression regulation. Despite the fact that RNAi is being widely applied in scientific and medical fields as a powerful tool, its mechanisms are not yet well understood. Components participating in this process have been thoroughly studied [2–9]. In contrast, knowledge concerning negative regulation for RNAi is poor.

The first clue of the existence of negative regulation of RNAi came from a plant virus. In tombousvirus, the p19 protein was found to specifically bind duplex siRNAs and block their assembly into RISC (RNA-induced silencing complex) [10,11], thus dampening the silencing of the RNAi. Another example of RNAi suppressor is the Hepatitis C Virus (HCV) core protein, which suppresses the activity of Dicer, which processes dsRNA into siRNA, and thus disturbs the cellular RNA silencing machinery [12].

Negative regulation of RNAi is a newly arising research area. The *Eri-1* gene encoding an exonuclease was discovered in a genetic screen for *C. elegans* mutants with enhanced RNAi efficiency. Mutations of this gene led to increased uptake of siRNAs [13]. Most recently, Fischer et al. reported their findings of trans-splicing in *C. elegans*, which generates the negative RNAi regulator ERI-6/7 [14]. In mammalian cells, ADARs (adenosine deaminases acting on RNA) have been reported to convert adenosine (A) into inosine (I), thus impairing the complementarities between dsRNA and the target mRNA. In addition, ADARs destabilize edited dsRNA, resulting in decreased efficiency of processing this molecule into siRNA by Dicer [15–18]. More interestingly, RNAi negative regulation seems to respond to the amount of RNAs entering the cell. We recently found an inverted correlation between the half-life of RNAi and the dose of siRNA both in cultured cells and in mice [19,20]. RT-PCR analysis further revealed increase of *adar-1* and *meri-1* mRNA induced by injected target esiRNA (endoribonuclease-prepared short interfering RNA) in a dose-dependent manner. More than 4-fold increase was reached upon intravenous injection of 10 μg esiRNA for each mouse. However, injection of 1 μg esiADAR-1 together with 10 μg of esiHBVP (esiRNA corresponding to DNA region encoding P protein of Hepatitis B Virus) did not alter the mRNA levels of *adar-1*.

In this study, we aimed to identify *cis*-elements responsible for the siRNA-induced transcription of *adar-1* in the promoter region and possible *trans*-factors binding to these *cis*-elements.

Abbreviations: RNAi, RNA interference; esiRNA, endoribonuclease-prepared short interfering RNA; esiHBVP, esiRNA corresponding to DNA region encoding P protein of Hepatitis B Virus; adar-1, adenosine deaminase acting on RNA, isoform 1; NP siRNA, non-specific siRNA; NP dsDNA, non-specific double-strand DNA.

☆ This study is supported by the Shanghai Leading Academic Discipline Project (no. B111), and NSF of China (no. 50533010).

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doi:10.1016/j.ijbiomac.2009.04.021

2. Materials and methods

2.1. Construction of plasmids

2.1.1. Construction of reporter plasmids

The 2 kb promoter region of the *adar-1* gene was amplified from genomic DNA of C57 mice. For construction of GFP reporter plasmids, a series of promoter segments were generated in a second round of PCR with various forward primers and a universal reverse primer as listed in Table 1. The forward primers contain an EcoRV and an AseI site, while the reverse primer contains a BamHI site. These amplified DNA fragments were cloned into pBluescriptII KS using restriction sites EcoRV and BamHI. After confirmation of the construction by means of sequencing, each of the promoter fragments was transferred into pEGFP-N1 vector (Clontech, USA) using restriction sites AseI and BamHI.

For construction of secreted alkaline phosphatase (SEAP) reporter plasmids, the forward primers for the second round of PCR contain SalI and BglII sites while the universal reverse primer contains EcoRI and NruI sites. The resulting fragments were first cloned into pBluescript II KS using restriction sites SalI and EcoRI. After DNA sequencing, these promoter fragments were transferred into pSEAP2-control (Clontech) with restriction sites BglII and NruI.

2.1.2. Construction of mammalian expression plasmid of PU.1

Full-length mouse *PU.1* cDNA was PCR-amplified with a forward primer (cggaattcagatgttacaggcgtgcaaaatgg, with EcoRI site) and a reverse primer (gaagatctctagtggtggtggtggtggtggtg, with BglII site). The PCR product was cloned into a pBluescript II KS using the EcoRI and BglII sites. After verification of the sequence, the fragment was transferred into pCMV-HA (Clontech, USA) to obtain the plasmid expressing PU.1, named pCMV-HA-PU1.

2.2. Preparation of PU.1 gene-specific esiRNAs and control non-specific esiRNAs

A DNA fragment containing exons 4 and 5 of the mouse *PU.1* gene was PCR-amplified using forward primer (cggaattcggatccgtttcctacatgccccgg, with EcoRI site) and reverse primer (gctctagatcagtggggcgggaggcgccgctc, with XbaI site), and cloned into pBluescript IIKS with corresponding restriction enzymes. After sequencing, the DNA fragment was transferred into a dual promoter plasmid pET-2P, which was derived from pET-22b to contain an extra *tac* promoter in opposite to the T7 promoter [21]. The resulting plasmid pET2P-PU.1 was transformed into the *E. coli* strain BL21 (DE3) to produce double-stranded RNA of the DNA fragment under the induction of IPTG (Isopropyl β-D-1-thiogalactopyranoside). Preparation of esiRNA of *PU.1* was carried out according to the method described by Xuan et al. [21], and the derived product was designated as esiPU.1.

Non-specific esiRNAs were derived from the Hepatitis B Virus P protein and prepared according to the lab protocol previously described [22], designated as esiHBVP.

2.3. Cell culture and transfection

CHO (Chinese Hamster Ovary) cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Gibco, USA), 100 μg/ml streptomycin and 100 IU/ml penicillin at 37 °C and 5% CO2. Cells were seeded at a density of 2×10^5/per well in 24-well plates and cultured overnight or to 90% confluence. Each well was then transfected with 0.4 μg of reporter plasmids, together with either 0.4 μg esiHBVP (non-specific esiRNA prepared from the DNA region encoding the P protein of HBV), or 0.4 μg non-specific 21-bp dsDNA, by using Lipofectamine 2000 (Invitrogen, USA). After transfection for 24 h, the expression of reporter genes was measured.

2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

Forty-eight hours after transfection, total cellular RNA was extracted using Trizol (Invitrogen, USA) according to the manufacturer's protocol. Reverse transcription was performed using Reverse Transcriptase XL (AMV) (Takara, Dalian, China). RT-PCR of both β-actin and *PU.1* fragments with different primer concentrations were amplified in the same tube. The primers used for *PU.1* amplification (atgttacaggcgtgcaaaatggaagggttttc and gggcatgtaggaaacctggtgactgaggccggtg) were used at 0.2 μM, whereas the primers for β-actin amplification (gagaccttcaacaccccagc and ccacaggattccatacccaa) were used at 0.02 μM. Amplification was carried out for 30 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 20 s. The PCR products were analyzed on a 1.5% agarose gel.

Table 1
Primers used for construction of report plasmids.

Reporter plasmid	Primer sequence	Promoter segment
GFP series		
pEGFPN1-2k	5′-cggatatcattaattcctgaaagcctcccagatta	(−2000)–(−1)
pEGFPN1-600	5′-cggatatcattaattacccagggatatggaagt	(−600)–(−1)
pEGFPN1-200	5′-cggatatcattaatgagtggtgaggaaggcggtg	(−200)–(−1)
pEGFPN1-180	5′-cggatatcattaatggcgtggactacggcg	(−180)–(−1)
pEGFPN1-160	5′-cggatatcattaatagcccttatggtgggc	(−160)–(−1)
pEGFPN1-140	5′-cggatatcattaattgctgctgctcggctacc	(−140)–(−1)
pEGFPN1-100	5′-cggatatcattaatttggcccaacagttggg	(−100)–(−1)
pEGFPN1-200ΔPU1	5′-cggatatcattaatttggcccaacagttggg	(−200)–(−1), ΔPU1
pEGFPN1-180 + PU1	5′-cggatatcattaattgaggaaggggcgtggac 5′-cgggatccagtgccggcaaggcccgcac[a]	(−180)–(−1), +PU.1
SAP series		
pSEAP2-2k	5′-cggtcgacagatcttcctgaaagcctcccaga	(−2000)–(−1)
pSEAP2- 600	5′-cggtcgacagatcttacccagggatatggaag	(−600)–(−1)
pSEAP2-200	5′- cggtcgacagatctgagtggtgaggaaggc	(−200)–(−1)
pSEAP2-180	5′- cggtcgacagatctggcgtggactacggcg	(−180)–(−1)
pSEAP2-160	5′- cggtcgacagatctagcccttatggtgggc	(−160)–(−1)
pSEAP2-140	5′- cggtcgacagatcttgctgctgctcggctac	(−140)–(−1)
pSEAP2-100	5′- cggtcgacagatctgagtggcggtgggcgtg	(−100)–(−1)
pSEAP2-200ΔPU1	5′- cggtcgacagatctgagtggcggtgggcgtg	(−200)–(−1), ΔPU1
pSEAP2-180 + PU1	5′- cggtcgacagatcttgaggaaggggcgtgg 5′-cggaattcgcgaagtgccggcaaggcccgcac[a]	(−180)–(−1), +PU1

[a] The reverse primers are bolded and indicated.