RESEARCH ARTICLE

APRICOT-1-2-3, A VERSATILE APPROACH TO COST-EFFICIENT GENES RNA INTERFERENCE USING ARTIFICIAL DUAL PROMOTER CASSETTE SYSTEM

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ABSTRACT

We have developed an artificial dual promoter cassette system called APRICOT-1-2-3, in which a target gene specific sequence is inserted between inverted two short artificial promoters. A single step PCR protocol has been developed by using this system that allows the production of this cassette in a high throughput manner. Actually, easily prepared APRICOT-1-2-3 constructs induced effective protein expression-silencing for p38MAPK and JNK. The flexibility of this unique design system suggests that additional modification can also easily be introduced into this siRNA expression cassette to improve the convenience applying RNAi.

INTRODUCTION

RNA interference (RNAi) can mediate sequence specific post transcriptional gene silencing and has been widely used to investigate gene functions, biological pathways, and the physiology of diseases. In mammalian cells, this is accomplished by introducing small interfering RNAs (siRNAs), short hairpin RNAs (shRNAs), or artificial miRNAs into cells (Elbashir *et al.,* 2001; McBride *et al.,* 2008). It is reported that the shRNAs are expressed at considerably higher levels relative to artificial miRNAs, providing mechanistic insight to explain their increased potency (Boudreau *et al.,* 2008). The shRNAs or long double-stranded RNAs are cleaved by Dicer to produce siRNAs (Zamore *et al.,* 2000; Bernstein *et al.,* 2001). Together with R2D2 (a small RNA binding protein), dicer also facilitates the formation of RISC (RNA-induced silencing complex), which in mammalian cells minimally contains a short, single-stranded RNA and a protein Argonaute2 (Tomari *et al.,* 2004; Liu *et al.,* 2004). Finally, RISC binds and cleaves the target complementary RNA (Hammond *et al.,* 2000). As we had fortuitously discovered the *human* Dicer gene for the first time (Matsuda *et al.,* 2000), using unique PCR-technique for detecting new genes (Matsuda *et al.,* 2000), we have been trying to search an easy cost-efficient and versatile approach applied for the RNAi induction. Here, we show an artificial dual promoter cassette system in which a synthetic DNA encoding a gene specific sequence is inserted between inverted two short artificial mammalian promoters. This system is designated APRICOT-1-2-3 for applauding RNA interference, saving cost and time, using the gene cassette through a one-step PCR, containing dual promoter activity, constructed with short three primers.

RESULTS AND DISCUSSION

The APRICOT-1-2-3 construct uses two inverted artificial minimal human promoters to independently transcribe the sense and antisense strands of the target siRNA, which will anneal to form a functional siRNA duplex. Schematic illustrations are shown in Figure 1A. The target sequence has no transcription termination signal. Probably, this artificial promoter sequence may be too short to produce long terminal ends of transcription by RNA-polymerase moving away. This is important hypothesis. The H1, U6, or other type3 promoters, which need the stop-sequences for ending RNA-transcription, are long enough to construct expression material by efforts and costs, or by insertion into expression-vector plasmid. Although the H1 promoter is about 300 bp in length, only four promoter elements, an unusual compact promoter in length, have been shown to be essential (Myslinski *et al.,* 2001). We improved this minimal promoter. The length was greatly reduced to about 55 bp, by rearranging the elements of mammalian promoter such as TATA box and/or CAAT box.

To produce the siRNA expressing cassettes, a single step PCR protocol was developed based on the dual promoter elements, and the procedure for synthesizing cassette by PCR is schematically depicted (Figure 1B). Inverted target RNAi sequence was used of 19 bp in length.

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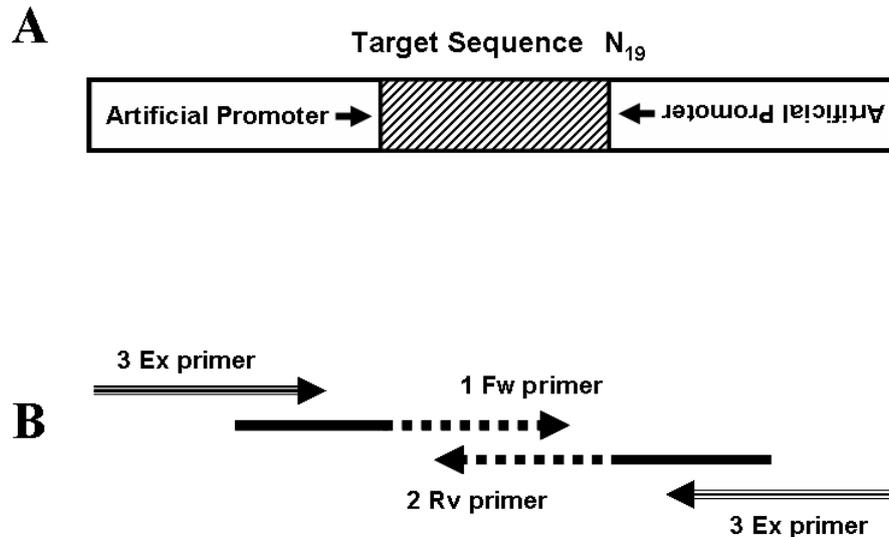E-mail: smatsuda@cc.nara-wu.ac.jp

Fig. 1. Artificial promoter expression cassette design for siRNA production. (A) Schematic representation of the APRICOT-1-2-3 DNA cassette. Note that artificial promoters are identical and about 55 bp in length, but inverted arranged on both sides of the target RNAi sequence, 19 bp in length. (B) Schematic representation of a single step PCR strategy for producing APRICOT-1-2-3 DNA cassette. To reduce cost in primer synthesis, the last 15 nt sequence within the Fw, Rv and Ex primers were designed as the annealing region. In the first several cycles, the two specific Fw and Rv primers were annealed through a complementary region of target sequences. The DNA fragment was simultaneously amplified for extension with Ex primer at the same time provided a proper annealing temperature for specific amplification. Note that 2 Ex primers (1 forward and 1 reverse) are identical for the sequences.
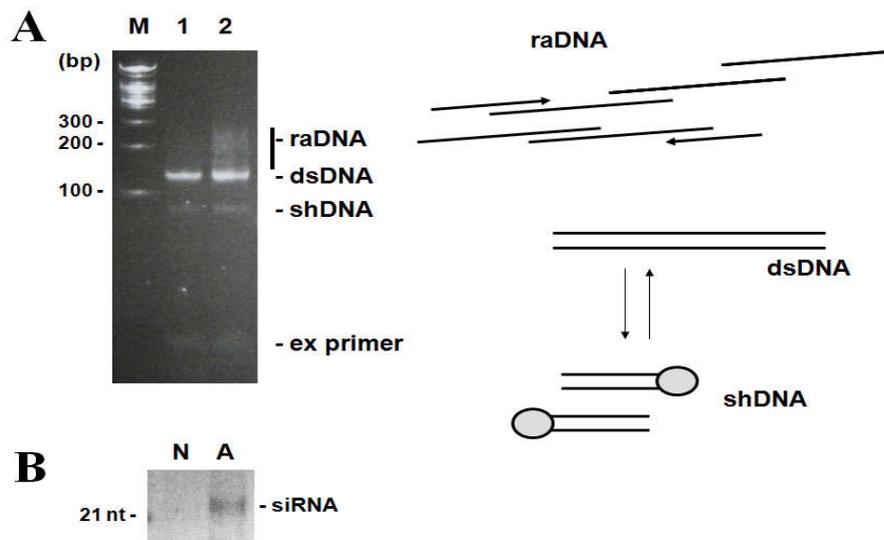

Fig. 2. (A) Results of the one-step PCR for APRICOT-1-2-3 construct. The primer extension and PCR reaction with KOD DNA polymerases were carried out as follows: 8 min at 94°C; 25sec at 94°C, 1min at 55°C, 35sec at 72°C for 8 cycles; followed by 25sec at 94°C, 1min at 30°C, 40sec at 72°C for 38 cycles (lane 1) and 48 cycles (lane 2). The Fw and Rv primers used are 1/40 amount of the Ex primer. The resulting PCR products were analyzed on 1.5 % agarose gel. M, 100 bp marker. 45 nt, 130 nt hairpin, 130 bp APRICOT-1-2-3 construct and upper smeared DNA fragments are indicated as ex primer, shDNA, dsDNA and raDNA, respectively. Prospected structure of these DNA fragments are schematically illustrated on the right. (B) Detection of siRNA expression. Small transcript Northern blot performed at 48 h post-transfection of an APRICOT-1-2-3 cassette (AP4 promoter for p38MAPK target) in HEK293 cells is shown. N, negative control with a scramble DNA. A, APRICOT-1-2-3 (AP4-p38MAPK).

The oligonucleotide primers encoding the target siRNA sequence and partial sequence of the artificial promoters (Fw primer, Rv primer) were used. In addition, an extension primer (Ex primer) complementary to the partial sequence of the artificial promoter and the remaining upstream promoter sequence was added to the PCR. The overlap-extended DNA fragment was then amplified by these three primers. To efficiently produce the desired PCR product, a PCR protocol was used that involved two separate cycling steps with annealing temperatures and with different amount of primers (Fw and Rv primers were used 1/40 amount of Ex primer). In the first 8 cycles, the three primers were annealed at 55 °C, then, in the following 38 cycles, the annealing temperature was decreased to 30 °C. If the reaction cycles were too high (48 cycles), it probably produces randomly annealed long DNA (raDNA) as seen smear bands in Figure 2A. And it could be reduced by lower annealing temperature and PCR cycles (Figure 2A). There is a possibility to improve the protocol including proper annealing temperature and primer amount so that the PCR results in amplification of only the full-length desired expression cassette. Under our favorite conditions at this moment, the 130 bp major PCR product as siRNA expression cassette is observed (Figure 2A). The Northern blot analysis using a probe complementary to target sequence comfirmed that APRICOT-1-2-3 construct,
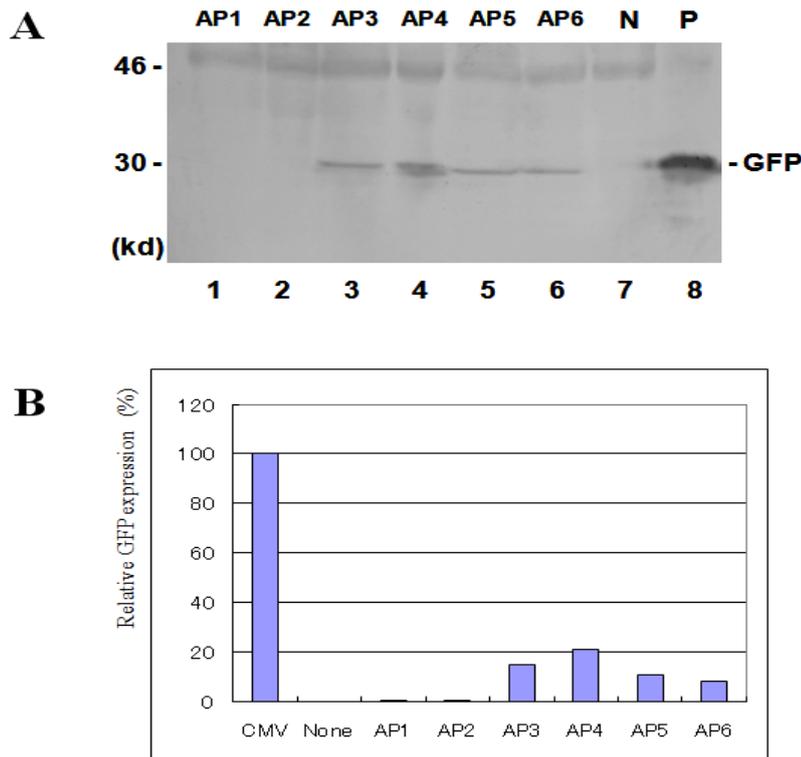
Fig. 3. Promoter activity of the artificial AP1, AP2, AP3, AP4, AP5, AP6 and CMV promoters. HEK293 cells were transfected with the 6 different promoter-candidates and CMV driven GFP (green fluorescent protein) expression plasmids (p-GLOW-TOPO, Invitrogen). The CMV-GFP plasmid (P) and promoter-less control vector plasmid (N) were used as a positive and a negative control, respectively. Upper panel: GFP expression level measured by Western blot with anti-GFP antibody (Santa Cruz). Lower panel: the values for relative GFP expression were shown as a percentage of expression level by CMV promoter.
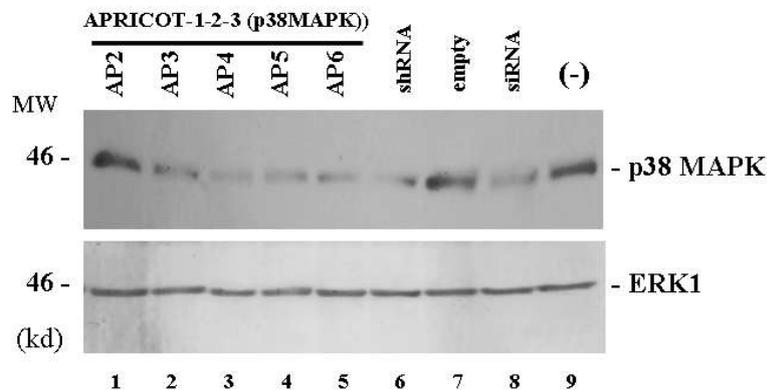

Fig. 4. (A) Comparisons in the levels of p38MAPK expression and silencing efficacy by promoter and construct dependent RNAi. HEK293 cells were transfected using Lipofectamine 2000 (Invitrogen) as directed by the manufacturer. 3 days after transfection with the indicated promoter driven APRICOT-1-2-3 constructs or control shRNA constract (pSilencer3.1-H1, Ambion), the p38MAPK expression was analyzed by Western blot with anti-p38MAPK antibody (Santa Cruz). (-), no transfectant parental cells as a negative control. empty, empty vector for shRNA expression. Erk1 protein expression measured with anti-Erk1 antibody (Santa Cruz) is also shown as equivalent loading controls. The maximal level of gene knockdown was observed with either functional promoter (AP3, AP4, AP5 andAP6) APRICOT-1-2-3 constructs or conventional shRNA constructs and siRNA. (B) Dose-response of APRICOT-1-2-3 inducible RNAi. HEK293 cells were transfected with 0.01, 0.05, 0.2, 1, 5, 20, 100 ng/ml (from lane 2 to lane 8, respectively) of APRICOT-1-2-3 construct (AP4 driven for JNK target). Western blot with anti-JNK antibody (Santa Cruz) revealed the suppression of endogenous JNK in response to the dose of construct. N, negative control with a scramble DNA. Erk1 protein expression with anti-Erk1 antibody (Santa Cruz) is also shown.

but not a scramble DNA construct, could produce siRNAs in trasfected cells (Figure 2B). In the method provided here, all three primers for synthesis of expression cassette are shorter than 55 nt in length. The cost and possibility of errors arising from synthesis of long oligonucleotides are therefore reduced.

To clarify whether the artificial promoter is really active to transcribe mRNA, the originated artificial promoter 6 candidates (designated as AP1, AP2, AP3, AP4, AP5 and AP6, all about 55 bp in length, detailed sequences not shown because of scheduling for patent-application)

were properly inserted into the upstream of GFP (green fluorescence protein) gene in GFP-expression vector plasmids, respectively. Among them, we successfully developed four independent artificial short expression promoters (AP3, AP4, AP5 and AP6) functional in the human-derived HEK293 cells as shown in Figure 3. To test whether the APRICOT-1-2-3 constructs with the active promoters (AP3, AP4, AP5 and AP6) would then functionally effective, expression-silencing against corresponding target genes was evaluated by transient transfection assays. Two genes encoding for p38MAPK

and JNK were selected as targets in order to validate the method. After purifying the PCR-products, these cassettes along with the positive and controls were transfected into HEK293 cells. Western blot analysis demonstrated that these APRICOT-1-2-3 constructs with the active promoters (AP3, AP4, AP5 and AP6), siRHA and shRNA which was produced from the original H1 promoter, but not with AP2 and empty vector cells, reduced endogenous p38MAPK protein expression in HEK293 cells (Figure 4A). Similar results were seen in human Huh7 liver cells (data not shown). We next introduced the most effective APRICOT-1-2-3 with AP4 for JNK-repression at various concentrations into HEK293 cells. Consistently, the JNK expression was reduced by the APRICOT-1-2-3 construct in a dose dependent manner (Figure 4B). The silencing efficiencies were also assessed by quantitative PCR following transfection of the siRNA expression cassettes (APRICOT-1-2-3 with AP4 and AP5 promoters for JNK-repression) into HEK293 cells. In agreement with our Western blot results, silencing of endogenous JNK mRNAs with those APRICOT-1-2-3s was also observed. We had detected no interferon-responses in introducing these APRICOT-1-2-3 constructs, when surveyed OAS1, OAS2, MX1, IFITM1 and ISGF3γ with interferon response detection kits (System Biosciences) at RT-PCR level.

The experiments demonstrated that the APRICOT-1-2-3 can mediate efficient RNAi. Moreover, the APRICOT-1-2-3 showed almost equivalent potency relative to conventional shRNA and siRNA in mediating gene silencing independent of target sequences. However, it was unclear whether this design was applicable to other target sequences and in different cell types. Furthermore, it remained unclear whether the APRICOT-1-2-3 expression draws out an infinitesimal interferon response as awkward side-effects in inducing RNAi (Gondai *et al.,* 2007; Bauer *et al.,* 2009). However, by using APRICOT-1-2-3 technology, RNA silencing of all arbitrary genes could be readily and simultaneously achieved, in cost and time saving manner. Modifying the promoter sequence to adjust modest transcription-level so as to disappear the interferon responses might be easily possible. To avoid vector instability that might be caused by two complementary promoter sequences flanking the siRNA encoding sequence (Zheng *et al.,* 2004), two different promoters, AP3 and AP4 for example, should be and could be chosen in the future experiments. The flexibility of the unique design of this construct suggests that additional modification of promoter sequence in order to adjust the RNAi on various cells without wicked responses can also easily be introduced into the siRNA expression cassette. More studies need to be undertaken to elucidate the precise molecular mechanisms of APRICOT-1-2-3.

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