Effect of β-globin MAR characteristic elements on transgene expression

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Abstract. The aim of the present study was to investigate the effect of the characteristic elements of matrix attachment region (MAR) on transgene expression. Human β-globin MAR was obtained by PCR amplification. A splicing MAR fragment containing all the characteristic elements of β-globin MAR was artificially synthesized and then cloned into the eukaryotic expression vector. Following digestion and sequence identification, we transfected Chinese hamster ovary (CHO) cells with the two vectors, and then screened for the transformation of stable cells. The transgene expression level was analyzed by ELISA, and the copy numbers of the CAT gene were analyzed by real-time fluorescent quantitative PCR. β-globin MAR enhanced CAT reporter gene expression by 2.1489-fold, whereas the β-globin MAR characteristic elements did not enhance this expression. The real-time fluorescent quantitative PCR analysis demonstrated that the relative copy numbers of the CAT gene of the β-globin MAR expression vector were 1.2-fold higher compared with those of the non-MAR expression vector. MAR was able to improve the transgene expression level to a certain extent. The MAR characteristic elements did not improve the transgene expression alone. The transgenic expression levels were not linear with the transgene copy number; however, the enhancement of transgenic expression was relative to the increase in the gene copy number.

Introduction

With the development of modern biotechnology, transgene technology has been widely used in genetic engineering and genetic therapy fields. However, low or silenced transgene expression has become a common phenomenon (1,2) that limits the use of transgene technology. Certain DNA elements have been used to overcome transgene silencing and to increase transgene expression (3,4), such as matrix attachment region (MAR) (5-7).

MAR is a unique DNA sequence capable of attaching to the nuclear matrix in eukaryotic chromatin. MAR is 300-1,000 bp long, and contains the topoisomerase consensus sequence and other AT-rich sequence motifs (8). Previous studies demonstrated that MAR is able to overcome transgene silencing, increase transgene expression and decrease the expression difference in different transformants, as a boundary regulatory element (9-11). In previous studies we demonstrated that human β-globin MAR is capable of efficiently enhancing transgene expression (12), and the transgene expression level may be highly increased when two β-globins flank the CAT expression cassette (13). Moreover, different MARs that flank the transgene are able to significantly increase the transgene expression level (14). As a cis-acting DNA element, MAR is capable of enhancing transgene expression, as it contains several characteristic motifs, such as the AATATATTT motif, the topoisomerase-II-binding site and an Alu element. However, the function of these motifs in enhancing gene expression, whether these motifs are able to enhance gene expression and identification of the MAR elements that are capable of increasing gene expression remain to be elucidated. In this study, we combined the characteristic elements of β-globin MAR, the AATATATTT motif, the topoisomerase-II-binding site and an Alu element, and removed the intermediate DNA connection for the synthesis of an artificial splicing of the MAR fragment to investigate the function and mechanism of the MAR characteristic elements in regulating transgene expression. Moreover, we examined the effect of the characteristic elements of β-globin MAR on transgene expression.

Materials and methods

MAR cloning and artificial synthesis. The fragment of human β-globin MAR (Genebank No. L22754) was amplified by PCR using the primers: P1: 5'-ATCGGTACC

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GTAAGACATCACCTTGCATTT-3' and P2: 5'-GCAACGCG
TCATAGTTTGAGTTACCCTTT-3'. Human β-globin MAR,
~2.15 kb in length (site 840-2,998), was amplified using human
peripheral blood as a template. The *Kpn*I/*Bgl*II enzyme sites
(GTCAGACTC and AGCGGTACC) with three additional
nucleotides were inserted at the 5' site of the above primers,
respectively. The PCR conditions were: 95°C for 3 min, 94°C
for 40 sec, 56-60°C for 30 sec, 72°C for 2 min at two cycles
every annealing temperature, 20 cycles at 55°C and a final
extension at 72°C for 5 min.

The characteristic elements of the human β-globin
MAR, including the AATATATTT motif (sites 840-848,
1149-1157 and 1636-1644), the topoisomerase-II-binding sites
(1252-1266, 1456-1470) and an Alu element (site 2687-2998),
were sequentially synthesized, forming an artificial splicing
of the MAR fragment ~369 bp long [Takara Biotechnology
(Dailan) Co., Ltd, China]. The *Kpn*I/*Bgl*II enzyme sites
(GTCAGACTC and AGCGGTACC) were inserted at the two
ends of the fragment.

Vector construction. The 2.15 kb MAR and 369 bp MAR
splice were inserted into the pMD18-T-vector (Takara Bio,
Inc., Shiga, Japan). Following sequence identification, two
MAR fragments and a pCATG plasmid were cut with *Kpn*I and
*Bgl*II, and then ligated using the T4 DNA ligase, to generate
the pCATGM1and pCATGM2 vectors containing the 2.15 kb
MAR and 369 bp MAR splice, respectively. The constructs are
shown schematically in Fig. 1.

Cell culture and transfection. Chinese hamster ovary cells
(CHO; Institute of Laboratory Animal Sciences, China) were
inoculated in six-well plates at $2x10^6$ cells/well. After 24 h,
the cells were transfected with pCATG, pCATGM1 and
pCATGM2 vectors according to the manufacturer's instruc-
tions (Xiamen Sunma Biotechnology Co., Ltd. Xiamen,
China). Following transfection for 48 h, the cells were screened
in DMEM supplemented with 800 mg/ml G418 (Calbiochem,
La Jolla, CA, USA) for 10 days. The cells were then screened
with a maintenance concentration of 400 mg/ml G418 for
10 days. When the colonies of stably transfected cells had
formed, these were digested with 0.25% trypsin. Each group
of cells was diluted and single colonies were selected. The
colonies were then replanted in a culture bottle to continue the
culturing process. The cells were collected when their density
reached 80-90%.

CAT expression assays. Cells were collected, adjusted to the
same concentration of $1x10^6$ cells/ml by cell counting, and
then washed three times with pre-cooled PBS. Lysis buffer
(1 M; 5-fold diluted) was then added to the tubes. The tubes
were gently agitated, left to stand at room temperature for
30 min, and then centrifuged at 13,000 x g for 10 min at 4°C.
Approximately 200 μl supernatant was extracted and placed
into EP tubes. Negative and positive controls were set. CAT
content was assayed using a CAT ELISA kit (Roche Applied
Science, Indianapolis, IN, USA) according to the manufac-
turer's instructions.

Analysis of gene copy number. Real-time fluorescent quantita-
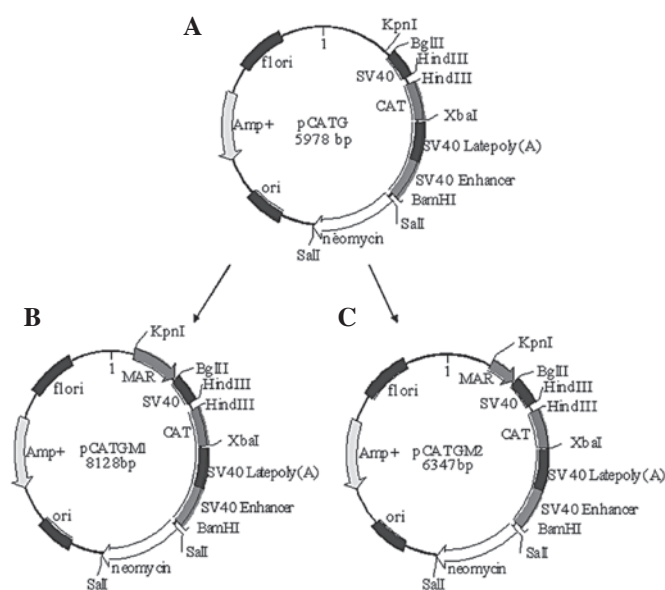tive PCR was used to analyze the gene copy number using

Figure 1. Plasmid vectors used in this study. Vectors are: (A) pCATG;
(B) pCATGM1 and (C) pCATGM2.

internal control primers as the controls. Stably transfected
cells were collected to extract the genomic DNA and served as
PCR templates. The primers were designed according to the
CAT gene sequence. The primers sequences used were: P1:
5'-CATCGCTCTGGAGTGAATACC-3' and P2: 5'-GGCATC
AGCACCTTGTCG-3'. The internal control β-actin primer
sequences were: P3: 5'-GTCTTTCTTCTGCCGTTCTC-3' and
P4: 5'-ACCAGCCTCATTAGGTTTGT-3'. The PCR amplifi-
cation system was 20 μl, and the PCR conditions were as
follows: 95°C pre-degeneration for 10 min, 95°C for 15 sec for
40 cycles, and 60°C for 1 min. The SYBR-Green I fluorescent
dye method was used for fluorescent quantitative real-time
PCR. Experimental data were treated with $2^{-\Delta\Delta Ct}$ (double ΔCt)
method. Each experiment was conducted in triplicate, and the
mean CT and ΔCt values [ΔCt = Ct (target gene) - Ct (internal
gene)] were calculated. The $2^{-\Delta\Delta Ct}$ was calculated, which is the
relative fold of a gene copy number to the internal control gene
copy number.

Statistical analysis. Analysis of variance was performed with
SPSS 17.0 (SPPS, Inc., Chicago, IL, USA). The Q-test was used
for comparison between two indices. P<0.05 was considered to
indicate statistical significance.

Results

Vector construction. The constructed vectors were digested
with *Kpn*I and *Kpn*I/*Bgl*II. The agarose gel electropho-
resis results are shown in Fig. 1. Following digestion of the
constructed pCATGM1 vector with *Kpn*I/*Bgl*II, an ~2.15-kb
fragment appeared. Following digestion of the constructed
pCATGM1 vector with *Kpn*I, linear DNA was evident (Fig. 2).
The results of the enzyme digestion conformed to the expected
fragments. The results demonstrated that the vector containing
MAR (2.15 kb) had been constructed. The same method was
used to analyze the constructed pCATGM2 vector; a 369-bp

Table I. CAT gene relative copy number analysis in stably transfected cell strains.

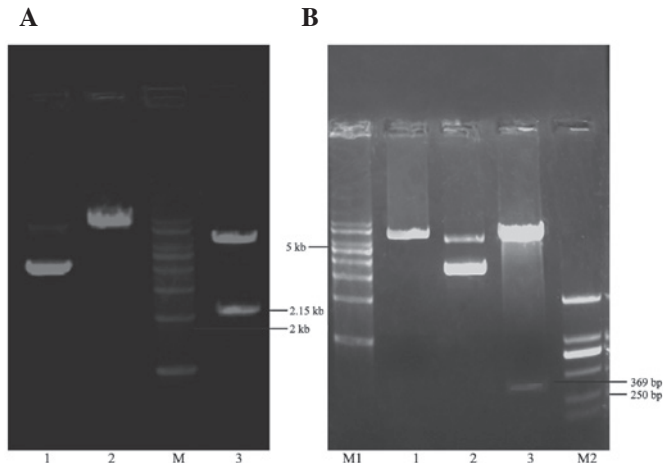|	Ct mean (vector)	Ct mean (β-actin)	ΔCt mean	ΔΔCt	Fold
pCATG	0.29277	20.94518	9.34759	0	1.0
pCATGM1	32.24645	23.20043	9.04602	0.30157	1.2

Figure 2. Identification of pCATGM1 and pCATGM2 using restriction enzymes. (A) pCATGM1 enzyme digestion. Lanes 1, no digestion; 2, *Kpn*I digestion; 3, *Kpn*I/*Bgl*IIdigestion; M, 1 kb DNA marker. (B) pCATGM2 enzyme digestion. Lanes 1, *Kpn*I digestion; 2, no digestion; 3, *Kpn*I/*Bgl*II digestion; M1, 1 kb DNA marker; M2, DL2000 DNA ladder.
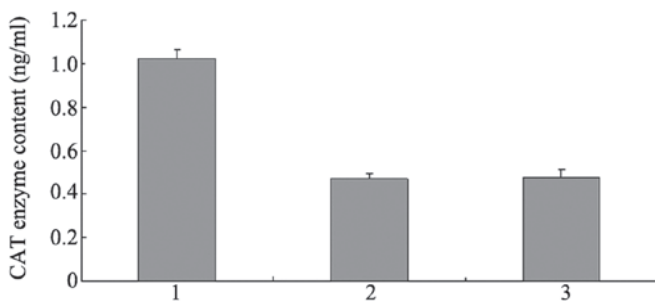

Figure 3. CAT content in different cells transfected with different vectors. Lanes 1, pCATGM1 plasmid; 2, pCATGM2 plasmid; 3, pCATG plasmid.

long fragment and linear DNA were observed. The vector containing MAR (369 bp) had also been constructed.

CAT assays. CHO cells were transfected with the constructed vectors using the non-MAR pCATG plasmid as control. The cells were screened by the use of G418. When the cell concentration reached 80-90%, the cells were adjusted to the same concentration of $1x10^6$ cells/ml via cell counting. The CAT content was analyzed following collection of the cells. The statistical analysis results from 10 monoclonal cells revealed that the CAT content of pCATGM1-transfected cells was 2.1489-fold higher than that of non-MAR pCATG-transfected cells. However, pCATGM2-transfected cells had the same CAT content as the non-MAR pCATG-transfected cells (Fig. 3). The difference in CAT expression between the different vector-transfected cells was statistically significant (P<0.01).

Gene copy number. Genomic DNA was extracted from six strains of cells from each group. Following real-time fluorescent quantitative PCR, $2^{-ΔΔCt}$ was calculated. Combined with ELISA analysis, the data from Table I demonstrate that the CAT gene copy numbers of the pCATGM1 expression vector were 1.2-fold higher compared with those of the pCATG expression vector (P<0.01). In addition, the gene expression of the pCATGM1 expression vector was 2.1489-fold higher compared with that of the pCATG expression vector. Therefore, the CAT gene expression of the different strains of the pCATGM1 expression vector was not linear with its gene copy number. However, exogenetic gene expression enhancing may be correlated with the increase in gene copy number.

Discussion

Studies with regard to MAR increasing transgene expression have been reported (6,15,16). Kim *et al* (9) inserted different MAR sources into the β-galactosidase gene expression vector and found that human β-globin MAR enhances the frequency of the positive colonies by 2.5-fold, and the β-galactosidase gene expression by 7-fold. However, other MAR sources have demonstrated little or no effect on β-galactosidase gene expression. Wang *et al* found that CAT gene expression was enhanced by 5.49-fold when human β-globin MAR was inserted in the 5' end of the CAT expression cassette (12). However, CAT gene expression decreased by 0.62-fold when the β-globin MAR was inserted at the 3' end of the CAT expression cassette. When the two ends of the CAT expression cassette were also inserted into the β-globin MARs, the CAT gene expression was 10.4-fold higher than that of the non-MAR expression vector (13). Wang *et al* found that if the two ends were inserted in the β-globin MAR and β-interferon MAR, the CAT gene expression was 4.5-fold higher than that inserted only with β-globin MARs at the two ends of the CAT expression cassette, and 46.4-fold higher than that of the non-MAR expression vector (14). Due to the different sources of MAR, different effects of MAR were observed. Certain MAR sources have a strong effect on gene expression, whereas other sources have little effect. Another reason for the different effects may be the site of the MAR insertion. The 5' end insertion possibly has more function than the 3' end insertion.

In this study, we inserted longer β-globin MAR, containing connection DNA and all the characteristic elements in the 5' end of the CAT expression cassette, thereby constructing the pCATGM1 expression vector. Following the transfection of CHO cells, we found that the pCATGM1 vector was capable of increasing CAT gene expression; the expression was 2.15-fold higher than that of the non-MAR expression vector. Therefore, human β-globin MAR is capable of enhancing the transgene

expression and decreasing transgene silencing. Our results are consistent with those of Kim *et al* (9). The pCATGM2 vector, which only contained characteristic elements, was not able to increase the CAT gene expression, similar to that of the non-MAR expression vector. These results confirmed that although the characteristic elements of MAR may have an important effect on gene expression enhancing in theory, the characteristic elements of MAR alone do not have the same effect as that of the MAR sequence. Thus, more studies are required to identify which characteristic elements of MAR are involved in gene expression enhancing and which have no effect, and to determine whether the connection DNA between MARs is necessary for gene expression enhancement. Although the characteristic elements of MAR may have an important effect on gene expression, the connection DNA between the characteristic elements is necessary for the shape-formation and function of MAR. If the connection DNA between the characteristic elements is missing, MAR is not able to form the correct shape, and thus is not capable of enhancing gene expression.

Real-time fluorescent quantitative PCR was used to analyze the gene copy number. The results revealed that the pCATGM1 vector was capable of increasing the gene copy number by 1.2-fold. In addition, β-globin MAR was able to enhance the transgene copy number. However, the CAT gene expression of different strains of the pCATGM1 expression vector was not linear with the gene copy number of the pCATGM1 expression vector. Thus, we deduced that exogenetic gene expression enhancement may be correlated with the increase in gene copy number. This result is not consistent with those of other studies (11,12). Therefore, the mechanism requires further investigation.

An increasing number of separated MAR fragments has led to an improvement in our understanding of the function and mechanism of MAR. However, investigations concerning MAR are in their infancy. Although several studies have shown that MAR is able to increase gene expression, decrease transgene silencing and decrease the expression difference in different transformants, knowledge of MAR expression regulation at the chromatin level and the mechanism of its effect on transgene expression remains limited and should be investigated.

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