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Analysis of Fish Expression Vectors for Construction of Two MARs Expression Vector System in Fish Cell Line

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In previously study, we isolated several fish matrix attachment regions (MARs) capable of replicating the plasmid by itself. In this study, we construct a fish expression vector, pBaEGFP(+), containing mud loach β-actin promoter, EGFP as reporter gene, and SV40 poly A signal. To analyze the effects of matrix-attached ARS elements, the cloned ARS elements were inserted into the multicloning site of the fish expression vector respectively. The fish ARS containing constructs, pBaEGFP(+)-ARSs, were transfected into a fish cell line, BF-2. In long-terms expression in BF-2 cells, The intensity of EGFP in transfected cells with pBaEGFP(+)-ARS101 and pBaEGFP(+)-ARS223 reduced 10 days to 25 days and then was constant to 30 days after transfection, while that of the control vector without ARS element was basal level. The intensity of both constructs showed about 3-fold of the intensity compared with the control vector on 30 days after transfection individually. *E. coli* back-transformation analysis shows that pBaEGFP(+)-ARS223 and pBaEGFP(+)-ARS905 maintain in episomal state at least 30 days after transfection. The result indicates that both may be able to replicate the vector in BF-2 cell. Therefore, the matrix-attached ARSs enhancing expression of the reporter gene might be useful as a component of the expression vector for transgenic studies.

Key words : MAR, ARS, *Misgurnus mizolepis*, EGFP, BF-2 cells.

Introduction

Specific DNA sequences attached to the nuclear matrix are called as matrix attachment regions (MARs).

MARs constitute boundaries of independently controlled chromatin units and, under proper conditions, display a moderate transcriptional enhancer activity (Xu et al., 1989; Phi-Van et al., 1990; Klehr et al., 1991). However, a number of studies indicate that when a MAR is placed between an enhancer and a promoter element, the transcriptional activity of reporter genes is reduced (Stief et al., 1989). Furthermore, for some unknown reasons, two MARs flanking a minidomain do not enhance efficiently the expression of reporter genes in transient transfection experiments but only in stably transfected cell lines (Blasquez et al., 1989a; Stief et al., 1989; Allen et al., 1993; Poljak et al., 1994), in transgenic animals (Xu et al., 1989; Kellum and Schedul, 1991; Forrester et al., 1994) and plants (Breyne et al., 1992). Two MARs (from yeast ARS1) flanking the β-glucuronidase (GUS) reporter gene under the influence of the 35S promoter of the cauliflower mosaic virus led to 24-fold increase in expression in stably transformed tobacco cell lines; seven cell lines exhibited an expression level from 100- to 850-fold higher than control cell lines transfected with the nonMAR construct, whereas only a 2-fold increase in expression was observed with the MAR construct in transient expression assays (Allen et al., 1993). A genetic analysis in *S. cerevisiae* showed that although ARS and MAR functions are separable, plasmid loss in the ARS assay is greatly reduced when the two are juxtaposed in the same plasmid (Amati et al., 1990). A large fraction of the MARs identified in *Drosophila* also show ARS activity when assayed in

yeast (Amati and Gasser, 1990; Brun et al., 1990). Interestingly, all identified ARSs (a total of 27) were present on MAR-containing fragments, except two, which were adjacent to MARs. Moreover, the presence of an ARS on a DNA fragment appeared to be highly correlated with the strength of binding (Brun et al. 1990). These results suggested that the MAR and ARS activities appear to be correlated and this correlation defines a MAR subclass. When these studies were extended to mammalian cells, conflicting results were obtained. One study observed that HeLa DNA sequences that behave as ARS elements in yeast actually reside in the DNA loop fraction (Cook and Lang, 1984). Other studies concluded that such sequences exit in the MARs. (Sykes et al., 1988).

It is still unclear whether any of the *Drosophila* and human sequences that behave as ARS elements in yeast represent *bona fide* replication origins. In fact, mammalian origins of replication are only now beginning to be understood. Generally it is accepted that nascent strands start at preferred sites in the genome, it is not known how these sites are restricted and whether or not they are genetically defined replicators analogous to those that direct replication in prokaryotic and simple eukaryotic organisms. Obviously the current lack of understanding of the nature of mammalian replication origins has a negative impact on attempts to evaluate the role of MARs in the initiation process.

Definitely, MARs clones displaying a strong origin of replication or enhancer activity might be useful as gene transfer and expression vehicles for transfection of animal and plant cells, in transgenic experiments, or in providing tools for the episomal replication of foreign genes in animal and plant cells.

Thus, to construct fish expression vector system, we attempted to clone fish MAR with ARS function in budding yeast and isolated sixteen fish ARSs from mud loach (Lim, 1999). In this study, several fish expression vectors were constructed using β-actin promoter from mud loach, EGFP gene as reporter gene, and various ARSs. We analyzed the effects of the MARs fragment with ARS elements on expression of reporter gene in fish cell line.

Materials and Methods

Strains and Plasmids

E. coli DH5αMCR (F⁻ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ 80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *deo*R *rec*A1 *end*A1 *sup*E44 λ⁻ *thi*-1 *gyr*96 *rel*A1) was used as a host for the transformation with plasmid DNAs. β-actin promoter of mud loach kindly provided by Dr. Chul Geun Kim in Hanyang University and pEGFP-1 (CLONTECH, Inc.) were used in the construction of pBaEGFP(+) to test ARSs effects on expression of EGFP reporter gene in fish cell lines. A fish cell line, BF-2 (a purchase from American Type Culture Collection) was used as host cells for analysis of ARSs effects.

Cell cultures

E. coli was grown in Luria-Bertani broth (LB; 1% Bacto-tryptone, 1% NaCl, 0.5% yeast extract) medium at 37℃ with vigorous shaking. When necessary, ampicillin was added in the LB to a final concentration of 50μg/mℓ. BF-2 (Bluegill fry, *Lepomis macrochirus*; ATCC CCL-91) cells were grown in Hank's balanced MEM (M-0643, Sigma) supplemented with 10% heat-inactivated fetal bovine serum (HyClone), 0.11 μg/mℓ sodium pyruvate, 1.5 mg/mℓ sodium bicarbonate, 100 units/mℓ penicillin, and 100 μg/mℓ streptomycin at 20℃.

Fish expression vector construction

To construct a fish expression vector, first pBaEGFP was constructed by inserting 1.6-kb mud loach β-actin promoter fragment into the site of Sma Ⅰ in pEGFP-1 and digested with Hind Ⅲ, and the 1.2-kb Hind Ⅲ digested fragment contain β-actin promoter was isolated and blunted. The expression vector, pBaEGFP(+), was constructed by ligating 1.2-kb blunted-HindⅢ fragment containing β-actin promoter and EGFP gene as reporter gene at the site of Ssp I/Nae I in pBluescript Ⅱ(+). Finally, constructions of matrix-attached ARSs containing pBaEGFP(+)-ARSs were carried out by ligating the ARSs at the site of EcoR I in pBaEGFP(+) (Fig. 1).

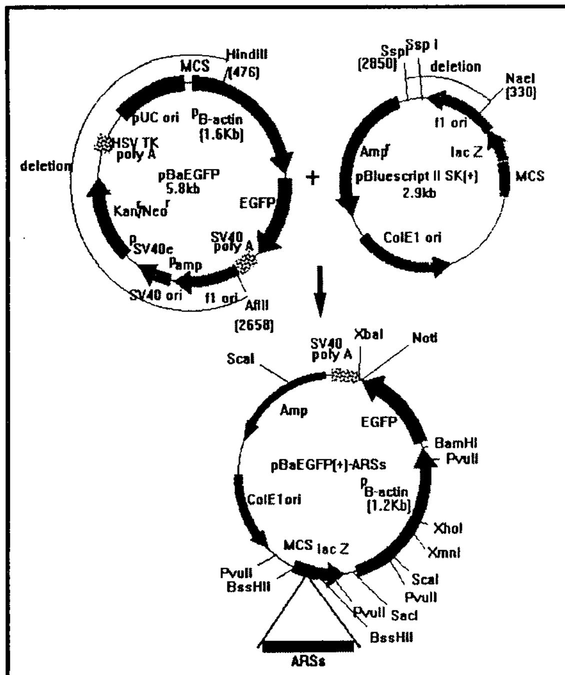

Fig. 1. Construction of pBaEGFP-ARS. pBaEGFP was constructed by inserting 1.6-kb mud loach β-actin promoter with intron 1 into the site of Sma I in the promoter cloning vector, EGFP-1. The construct was digested with Hind III and Afl II, isolated the 2.2-kb Afl II/Hind III fragment containing β-actin promote (1.2 kb), EGFP gene, and SV40 poly A. The digested fragment was gap-filled using T4 DNA polymerase. A fish expression vector, pBaEGFP(+), was constructed by ligating 2.2-kb Afl II/Hind III fragment at the site of Ssp I/Nae I in pBluescript II (+). Finally, pB-aEGFP(+)-ARSs were constructed by inserting ARSs into multicloning sites of the pBaEGFP(+).

Transfection of fish cell lines

Transfection of fish cell line, BF-2 was carried out using the electroporator (BTX, electorsquare porator T820). Fish cell lines were grown to a density of 80% confluency, trypsinized, and centrifuged at 300 × g for 3 min. The pellet was suspended in ice-cold phosphate-buffered saline (without added $MgCl_2$ and $CaCl_2$) and centrifuged again. The pellet was resuspended in cold phosphate-buffered saline (without added $MgCl_2$ and $CaCl_2$) at a concentration of 1.5×10^7 cells per mℓ. 15 ㎍ of plasmids was added in 400 ㎕ of cell suspension. The mixture of cells and DNA were loaded between the electrodes. The cuvette (4 mm gap) was kept on ice for 10 min. After pulse (1.25 kV, 1msec, pulse number 4), 1 mℓ of media was immediately added before being added to 10 mℓ of growth medium. The transfected cells were cultured until the appropriate time of reporter gene assay without stringency.

Measurement of enhanced green fluorescent protein (EGFP) expression

The growth medium was removed from the cells and rinsed twice with PBS. 1 mℓ of 1 × lysis buffer (100 mM potassium phosphate pH 7.8, 0.2% triton × -100, 1 mM DTT) was added and leaved for 5 min. The suspension was transferred to 1.5 mℓ tube and vortexed for 1 min. The lysate was placed on ice for 45 min, centrifuged at 12,000 × g for 5 min, and transferred the cleared lysate to a 1.5 mℓ tube.

The fluorescence intensity was measured in Luminescence spectrometer LS 50B (PERKIN ELMER) using an excitation filter of 488 nm and an emission filter 510 nm.

DNA preparation from transfected fish cell lines

DNA was isolated from transfected BF-2 cells using the Wizard genomic DNA purification Kit (Promega). The cells were harvested after trypsinization and transferred to a 1.5 mℓ tube. Cells were centrifuged for 10 sec at 13,000 ~ 16,000 × g to pellet the cells. Supernatant was removed and added of nuclei lysis solution into the pellet, pipeted up and down to lysis the cells, added 3 ㎕ of RNase solution (4 mg/mℓ) into the sample and incubated to 15~30 min at 37℃. 200 ㎕ of protein precipitation solution was added and vortexed vigorously at high speed for 20 sec. After centrifugation for 3 min at 13,000~16,000 ×g, supernatant containing the DNA was transferred to clean 1.5 mℓ tube containing 600 ㎕ of isopropanol. This sample was gently mixed and then carried out the centrifugation for 10 min at 13,000~16,000×g. The supernatant was decanted and then 600 ㎕ of 70% ethanol was added to wash DNA. The DNA was pelleted by centrifugation at 13,000~

16,000×g at room temperature for 5 min. Using aspirator ethanol was aspirated then the pellet was dried. 100 μℓ Tris-EDTA solution was added and incubated at 65℃. The DNA was used for *E. coli* back-transformation and polymerase chain reaction (PCR).

Southern hybridization

Total fish DNAs were isolated from transfected BF-2 cells on 30 days after transfection as described above. The DNAs were back-transformed into *E. coli* and the plasmids were rescued from the bacteria. These rescued plasmids were used for southern hybridization. Probe DNAs were labeled with DIG-11-dUTP using DIG Nucleic Acid Labeling Kit (Boehringer Mannheim) and southern hybridization were performed as followed; Gels were run in the presence of ethidium bromide at 1-2 V/cm in TAE buffer (0.04 M Tris-acetate pH 8.3, 1 mM EDTA). The DNA was transferred to GeneScreen Plus (Dupont-NEN Research Products) in 0.4 M NaOH for 4 h. The positively charged nylon membranes were rinsed in 2×SSC (300 mM NaCl, 30 mM sodium citrate, pH 7.0). After a 30 min of prehybridization the membranes were hybridized overnight at 68℃ in hybridization solution (0.75 M NaCl, 0.075 M sodium citrate, 2% (w/v) N-lauroylsarcosine, Na-salt (Sigma), 0.02% (w/v) SDS, and 1×blocking reagent) with DIG-labelled DNA. The membranes were washed 2 ×5 min at room temperature with at least 50 mℓ of 2×SSC, 0.1% (w/v) SDS, per 100 ㎠ membrane, 2× 15 min at 68℃ with 0.1×SSC, 0.1% (w/v) SDS, and 1 min in buffer 1 (100 mM Tris-HCl, 150 mM NaCl, pH 7.5). After incubation for 30 min 100 mℓ blocking reagent, the membranes were incubated for 3 min with 20 mℓ of diluted antibody conjugate solution, and washed 2×15 min with 100 mℓ wash buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5), and equilibrated for 2~5 min in 20 mℓ detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, 50 mM $MgCl_2$, pH 9.2). Exposure was made at room temperature in the dark. 10 mℓ freshly prepared color-substrate solution sealed in a plastic bag.

Polymerase Chain Reaction (PCR)

To detect vector constructs transfected into fish cells, template DNAs were isolated from BF-2 cells transfected with pBaEGFP(+) or pBaEGFP(+)-ARSs on 30 days after transfection. PCR was performed using EGFP primers (forward; CCC-ATC-CTG-GTC -GAG-CTG-GA, reverse; AGG-TAG-TGG-TTG-TCG-GGC-AGC). Thermocycling condition was as follows: 30 cycles of 1 min-denaturation at 94℃, 1 min-annealing at 60℃, and 1 min-extension at 72℃ with a final elongation step of 7 min at 72℃. To estimate PCR product yield and purity, 5 μℓ of amplified sample was electorphoresed through 1% agarose using TAE buffer.

Results

Construction of expression vectors containing matrix-attached ARS elements

To assay the effect of matrix-attached ARSs on expression of transferred gene into fish cell line, first, pBaEGFP was constructed by inserting 1.6-kb mud loach β-actin promoter into the Sma I site in the promoter cloning vector, pEGFP-1. The construct was digested with Hind III and Afl II, isolated the 2.2-kb Afl II/Hind III fragment containing β-actin promoter, EGFP gene, and SV40 poly A. The isolated fragment was gap-filled using T4 DNA polymerase. Second, a fish expression vector, pBaEGFP(+), was constructed by ligating 2.2-kb Afl II/Hind III fragment at the Ssp I/Nae I site in pBluescript II (+) after digestion with Ssp I and Nae I. Finally, pB-aEGFP(+)-ARSs were constructed by inserting the ARSs into multicloning sites of the vector (Fig. 1) and then transferred into a fish cell line, BF-2 by electroporation.

Transfection and the effects of ARS elements on the expression of reporter gene.

In recent studies various gene constructs are introduced into fish cell lines for transgenic fish study (Moav et al., 1992; Betancourt et al., 1993;

Zhang et al., 1998; Hahm et al., 1998). In this study a fish expression vector, pBaEGFP(+), was constructed and then transfected into BF-2 cells. The transfection showed that the 1.2-kb β-actin promoter of *M. mizolepis* was active in BF-2 cells (Lim, 1999). So, pBaEGFP(+)-ARSs and non-ARSs pBaEGFP(+) were transferred into BF-2 cells to assay the effects of ARS elements on expression of reporter gene in fish cell line.

The fluorescence intensity of EGFP increased until 7 or 8 days post-transfection and then reduced in transfected BF-2 cells. Thus in a transient expression assay, the relative fluorescence intensity was measured on 7 days after transfection. The relative fluorescence intensity of all tested constructs with ARSs, except the pBaEGFP(+)-ARS101, was slightly lower than that of the control (Lim, 1999). For a long-term expression assay, the intensity fluorescence was measured from 10 to 30 days post-transfection at every 5th day. The cells transfected with pBaEGFP(+)-ARS101 and pBaEGFP(+)-ARS223 showed that the expression of EGFP gene reduced gradually until 25 days post-transfection and then was maintained constantly to 30 days. These intensity were about 3-fold higher than that of control vector (Fig. 2). The results indicated that ARS101 and ARS223 enhanced the expression of reporter gene in BF-2 cells.

In case of transfected CHSE-214 with the control vector and pBaEGFP(+)-ARS905(E1158), the expression of the control vector showed like a typical transient expression pattern. In pBaEGFP(+)-ARS905(E1158), however, the intensity was continuously increased until 11 days. Then, the fluorescence intensity was higher about 7-fold than that of control vector (Lim, 1999). The overall data suggested that some of the constructed vectors might be useful as an element of the gene construct for transgenic fish studies.

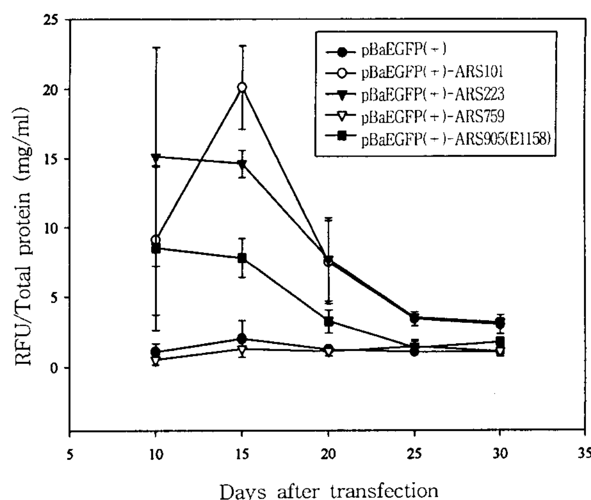

Fig. 2. Comparison of fluorescence intensity of EGFP in lysate of transfected BF-2 cells with pBaEGFP-(+)-ARSs relative to control pβaEGFP(+)-transfected cells lysate. BF-2 cells were transfected with pBaEGFP(+) and pBaEGFP(+)-ARSs by electroporation and the each cell lysate was prepared from cells incubated for 10, 15, 20, 25, and 30 days after transfection as described in the Materials and Methods. Fluorescence intensity was measured in Luminescence Spectrometer LS 50B (PERKIN ELMER) using an excitation filter of 488 nm and an emission filter 510 nm. Data show means of two separate experiments of duplicate determinations. The standard deviations from the mean are indicated by error bars.
RFU; Relative fluorescence units

Detection of pBaEGFP(+)-ARSs in transfected BF-2 cells

To determine whether the transfected vectors containing ARSs are maintained in an episomal state, DNAs were isolated from BF-2 cells transfected with pBaEGFP(+) or pBaEGFP(+)-ARSs on 30 days after transfection. *E. coli* back-transformation was performed with total DNAs prepared from the transfected cells. Interestingly, As shown in Fig. 3, the pBaEGFP(+)-ARS223 and pBaEGFP(+)-ARS905 were shown to be episomally maintained in transfected BF-2 cell lines at least 30 days after transfection. Alternatively, Fig. 4 shows the PCR analysis using DNAs prepared from BF-2 cells transfected with pBaEGFP(+) or pβaEGFP(+)-ARSs on 30 days after transfection. The EGFP reporter gene were detected in all the transfected cells but not untransfected cells. As shown Fig. 3, however, two ARSs of four were rescued from transfected BF-2 cells. The results indicate that the ARS223 and ARS905(E1158) can autonomously replicate the vector in BF-2 cells. The

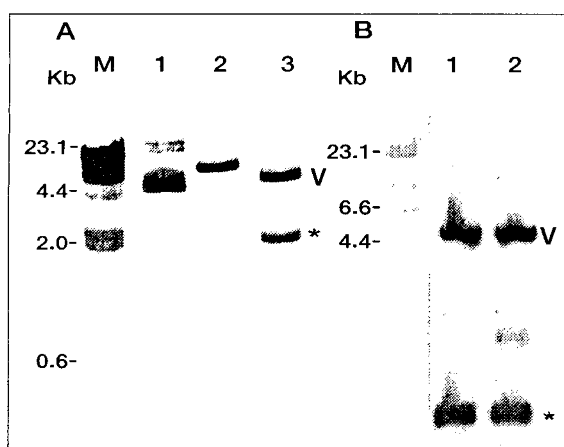

Fig. 3. Hybridization analysis of plasmids isolated from *E. coli* back-transformed with DNA extracted from transfected BF-2 cell line on 30 days after transfection. DNA was isolated from transfected BF-2 cells with pBaEGFP(+) and pBaEGFP(+)-ARSs on 30 days after transfection. *E. coli* back-transformation was performed with prepared DNA from the transfected cells. A. Isolated plasmids from *E. coli* back-transformed with DNA extracted from pBaEGFP(+)-ARS 223-transfected BF-2 cells were hybridized with DIG-labeled pBaEGFP(+)-ARS223 digested with EcoR I. V indicates pBaEGFP(+) digested with EcoR I. Asterisk (*) indicates inserted ARS individually.
M, λ-DNA digested Hind III;
1, the rescued plasmids from pBaEGFP(+)-ARS223-transfected BF-2 cells;
2, the rescued plasmids digested with Pst I;
3, the rescued plasmids digested with EcoR I.
B, Isolated plasmids from *E. coli* back-transformed with DNA extracted from pBaEGFP(+)-ARS905(E1158)- transfected BF-2 cells were hybridized with DIG-labeled pBaEGFP(+)-ARS905(1158) digested with EcoR I.
M, λ-DNA digested Hind III;
1, non-transfected pBaEGFP(+)-ARS905(E1158) digested with EcoR I;
2, the rescued plasmids from pBaEGFP(+)-ARS905 (E1158)-transfected BF-2 cells.

data suggested that mud loach matrix-attached ARSs are a useful element as a component of fish expression vectors in fish cell lines even though detailed analysis remains to be investigated.

Discussion

MARs were positioned near replication initiation sites in the amplified dihydrofolate reductase domain of Chinese Hamster Ovary cells (Dijkwel, P. A. and Hamlin, J. L. 1988). Sykes, et al. (1988) showed that yeast ARS function and nuclear matrix association were coincided in a short sequence from the human HPRT locus. Mah, et al. (1993) showed that ors 12, a mammalian autonomously replicating DNA sequence, was associated with the nuclear matrix in a cell cycle-dependent manner. In addition, Amati and Gasser, (1990) showed that two of the four *Drosophila* MARs have ARS in budding yeast, and three function in fission yeast. Although MARs did not share extensive sequence homology within or among species, they did display a number of significant similarities, the most obvious of which was an elevated A+T content.

In the present studies all cloned ARSs was asso-

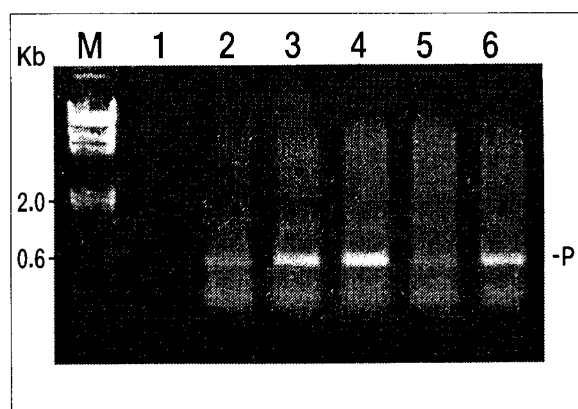

Fig. 4. PCR analysis of DNA extracted from transfected BF-2 cells on 30 days after transfection. Template DNAs were isolated from BF-2 cells transfected with pBaEGFP(+) or pBaEGFP(+)-ARSs on 30 days after transfection. PCR was performed using EGFP primers (forward primer, CCCATCCTGGTCGAGCTGGA; reverse primer, AGGTAGTGGTTGTCGGGCAGC). Thermocycling condition was as follows: 30 cycles of 1 min-denaturation at 94℃, 1 min-annealing at 60℃, and 1 min-extension at 72℃ with a final elongation step of 7 min at 72℃. P indicates 566-bp PCR products.
M, λ- DNA digested with Hind Ⅲ;
1, non-transfected DNA;
2. pBaEGFP(+)-transfected DNA;
3, pBaEGFP(+)-ARS101-transfected DNA;
4, pBaEGFP(+)-ARS223-transfected DNA;
5. pBaEGFP(+)-ARS759-transfected DNA;
6. pBaEGFP(+)-ARS905(E1158)-transfected DNA.

ciated with the nuclear matrix isolated from mud loach blood cells. This data suggested that ARS is included in a fraction of MAR. The cloned ARS with high integrated strengths of matrix association potential (> 100) showed high affinity with the nuclear matrix (Lim, 1999). In addition, The subclones of ARS905 with high transformation efficiency showed higher affinity binding to nuclear matrix. The data indicate that ARS function and matrix binding affinity appear to be correlated (Lim, 1999).

In the *Xenopus* rDNA locus, however, a random loop organization is found whether replication is initiated at random sites or specifically within the intergenic spacers. DNA replication initiates at random throughout these repeats, with no preference for the MAR, and at size interval that in both embryos (8 kb) and adult cells (25～50 kb) are clearly larger than the loop size (Shinomiya and Ina, 1993). Replication of he DHFR gene of CHO cells initiates over a broad 55-kb zone that contains a MAR. In the human β-globin gene cluster, replication only initiates between the β- and δ-globin genes (Kitsberg et al., 1993), while at least eight MARs have been identified in the cluster. These overall data indicate that replication origins and MARs coincide or are juxtaposed, eventhough does not initiate at all MAR, and can initiate at non-MAR sequences.

In mouse L cells, 15 muARSs were used for CAT expression vector. Relatively high copy number was found in most of the transformed cell-line (Holst et al., 1988). They suggested that amplification might occur by reinitiation of DNA replication with in the nuARS element. Hemann et al. (1994) used successfully the nuNTS1 element as amplification promoting element for secreted alkaline phosphatase in mouse L fibroblast. Even if these DNA element were not originated from MAR, the data suggest that fish ARS is a useful element for gene transfer and expression vehicles for transfection of fish cell lines. In addition, in transient expression a MAR enhanced expression of luciferase gene in fish cell line, CHSE-214 (Hahm et al., 1998).

MARs were proposed to sculpture the crossroads of the differential activation of origin (during development and S-phase) and of the control of gene expression and pattern formation in embryogenesis (Boulikas, 1992b). Thus, large-scale random cloning of MARs might advance our knowledge of the control of initiation of DNA replication and function of transcriptional enhancers as well as domain structuring of genomes during development. Furthermore, MAR clones displaying a strong origin of replication or enhancer activity might be useful as expression vectors in transgenic studies.

To evaluate effect of fish matrix-attched ARSs on expression of EGFP gene, several different pBaEGFP(+)-ARSs or a non-ARS control vector were transfected into BF-2 or CHSE-214 cell lines. For the transgenic study, the long-term expression assay were carried out. The comparison of expression activity of four vectors containing ARSs and a non-ARS control were appeared in Fig. 2. ARS101 and ARS223 enhanced the expression of reporter gene in BF-2 cells. As shown in Fig. 3, the pBaEGFP(+)-ARS223 and pBaEGFP(+)-ARS905(E1158) were shown to be episomally maintained in transfected BF-2 cell lines at least 30 days after transfection. The results indicate that the ARS223 and ARS905(E1158) can autonomously replicate the vector in BF-2 cells. This data indicates that all ARSs in yeast can not do active in fish system. It suggests that DNA replication machinery or its mechanism is relatively different between yeast and fish system. In additional transient expression of CHSE-214 cells, the expression ratio of pBaEGFP(+)-ARS905(E1158) showed higher efficient than that of control pBaEGFP(+) (Lim, 1999). Especially, ARS905(E1158) dramatically increased EGFP gene expression but not the control. It suggests that the increasing expression is due to function of ARS because ARS905(E1158) was episomally maintained at least to 30 days after transfection in BF-2 cells (Fig. 3).

The overall data indicate that the matrix-attached ARS might be useful as a component of expression vector for transgenic fish experiments, or provide the

tool for an episomal replication of foreign gene in fish cell lines.

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