Improved bicistronic mammalian expression vectors using expression augmenting sequence element (EASE)

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Abstract

We have recently reported on the isolation of a 5.7 kb segment of Chinese hamster ovary cell genomic DNA, Expression Augmenting Sequence Element (EASE), which when used in bicistronic expression vectors allows the development of stable Chinese hamster ovary cell pools in a five to seven week time period that express high levels of recombinant protein (6–25 $\mu\text{g } 10^{-6}$ cells/day depending on the protein). In the present study, we have mapped the activity of the EASE to a 2.1 kb region using colony forming assays and developed bicistronic expression vectors with the smaller EASE or control lambda DNA. The recovery of pools expressing the hematopoietic growth factor, FLT3 Ligand, in methotrexate-containing media took 1 to 4 weeks less when using EASE expression vectors compared with control vectors. The cell pools developed with the EASE and control vectors had similar final protein expression levels. Southern blot analysis suggested the expression cassette from the EASE containing vectors integrated in tandem arrays arranged in either head to head or head to tail fashion. By contrast, control vectors appeared to integrate with multiple interruptions to the expression vector. Thus, the EASE, within a bicistronic expression vector, appeared to facilitate tandem vector integration and reduce the time required to develop cell pools for protein expression.

Introduction

Bicistronic expression vectors are effective tools for expressing recombinant protein in host cell lines (Kaufman et al., 1987). Expression of recombinant protein from these vectors is made more stable with the use of a viral internal ribosomal entry site sequence (Kaufman et al., 1991). Typically, a bicistronic expression vector encodes an amplifiable selectable marker on the same transcript as a gene of interest. When these vectors are transferred into host cells and the cells are grown step wise under increasing selective pressure, the level of expression of the recombinant protein usually follows the expression of the selectable marker. To generate stable cell lines, cloning steps can then be performed.

The Expression Augmenting Sequence Element (EASE) improves expression of recombinant proteins from bicistronic vectors in Chinese hamster ovary (CHO) cells by decreasing the number of selective steps needed to develop pools that express high levels of recombinant protein (Morris et al., 1997). In the present study, we have further characterized EASE activity by mapping it to a 2.1 kb region of CHO genomic DNA. Expression vectors using the minimal EASE were compared with control vectors. Stable pools expressing recombinant protein could be developed faster using the EASE vectors, and recombinant protein expression was comparable. In addition, we found that EASE may act by improving integration and amplification of plasmid DNA.

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Figure 1. Constructs used for mapping study. Each vector contains the huCMV promoter/enhancer driving the expression of either a bicistronic mRNA encoding a gene of interest and murine DHFR or muDHFR alone. 5' to the huCMV promoter/enhancer various lengths of CHO DNA containing EASE activity or control DNA were inserted as indicated. Elements used in each construct are indicated in key at the bottom left. Dashed line represents deleted regions of CHO DNA. Restriction enzyme sites are indicated.

Materials and methods

Construction of plasmids

The manufacturers' instructions were followed for all enzymes used in plasmid constructions. Enzymes were purchased from New England Biolabs, Beverly, MA, Boehringer-Mannheim Biochemicals, Indianapolis, IN or Stratagene, La Jolla, CA. Plasmid pG.2 was constructed from plasmid pG5.7 (Figure 1 and Morris et al., 1997) as follows. The TNFrFc cassette was removed by digestion with *NotI* and ligation. The resulting plasmid was cut with *EcoRI* and *BbsI*, the ends filled with Klenow and ligated. To facilitate cloning of some of the constructs used for mapping studies, the *BbsI* site of pG.2 was replaced with a *BamHI* site. Plasmid pG.2 was digested with *BbsI*, the ends filled using *Pfu* polymerase and then a *BamHI* linker (New England Biolabs) added. This plasmid was named pG.2B. The 3.6 kb *EcoRI* fragment and the 1.8 kb *EcoRI-SmaI* fragment from pG5.7 were each cloned into pG.2B to make plasmids pG3.6 and pG.2SE1.8, respectively. In both cases, the ends of the fragments were filled and ligated to *BbsI* cut and filled pG.2. The 2.1 kb re-

gion of pG5.7 used to construct pG2.1 was amplified by PCR using the following oligonucleotides: 5'-GCGCGCGGATCCTACATTGTAATTCCTAAATTCTA-3' and 5'-AAAGGATCCGTTTCGTGGTGGACATCAC-3'. The PCR product was purified using the Wizard DNA Clean-Up System (Promega, Madison, WI). It was digested with *BamHI* and then cloned into the *BamHI* site of phagemid pBluescript II SK +/- (Stratagene.) Following dideoxy sequence analysis, the 2.1 kb *BamHI* fragment was removed from this intermediate construct and cloned into the *BamHI* site of pG.2B to create pG2.1. The 3.8 kb *BamHI* fragment from plasmid 2a5-3λ (Morris et al., 1997) was cloned into the *BamHI* site of pG.2B to make pG3.8. Plasmid pGλ was constructed by cloning the 2.0 kb *BamHI-BclI* fragment from bacteriophage lambda into the *BamHI* site of pG.2B. Plasmids used for protein expression studies were derivatives of pG2.1 and pGλ. A cDNA for FLT3L (Brasel et al., 1996), the encephalomyocarditis virus internal ribosomal entry site (nucleotides 260–824) (Jang and Wimmer, 1990) and the dihydrofolate reductase gene were cloned into the *NotI* and *SalI* sites of pG2.1 and pGλ to create pG2.1FLT3L and pGλFLT3L, respectively (Figure 2).

Figure 2. Mapping of EASE activity to a 2.1 kb region of CHO genomic DNA. Each data point represents results from 5 or 6 independent transfections for each plasmid. Colony forming assays were performed in 10 nM MTX. Colony formation is shown relative to results for pG3.6. Error bars represent standard error of the mean.

DXB-11 cell culture, transfection and colony forming assay

DXB-11 CHO cells were maintained in nonselective GHT medium (DMEM:F12 supplemented with 7.5% bovine calf serum [Hyclone, Logan, UT], 2 mM L-glutamine, 90 μ M thymidine [T], 90 μ M hypoxanthine [H] and 120 μ M glycine [G]) in a 37 °C humidified incubator containing 5% CO₂. Transfections by electroporation were performed as follows. For each electroporation, 10 μ g of plasmid DNA was linearized using an appropriate single cutting enzyme. Restriction reactions were dialyzed and the DNA quantitated again prior to transfection. Mid-log phase DXB-11 cells were harvested, washed once with cold electroporation buffer (20 mM HEPES, pH 7.4, 137 mM NaCl, 5 mM KCl, 0.7 mM Na₂HPO₄, 6 mM dextrose) and then resuspended in electroporation buffer at 10⁷ cells per mL. Linearized DNA (5–10 μ g) was added to 4 $\times$ 10⁶ cells. After 5 min on ice, the cells were electroschocked using a BTX Electro Cell Manipulator 600 (San Diego, CA) at 500 V, 400 μ F, 13 ohms and charging voltage of 174. The cells were returned to ice for a 10 min incubation before plating.

Figure 3. Plasmids used to test the activity of EASE in stable pools. Adeno = Adenovirus tripartite leader sequence, FLT3L = cDNA coding for FLT3L, IRES= internal ribosomal entry site, DHFR = cDNA coding for murine DHFR. Arrow indicates the direction of transcription and structure of the bicistronic mRNAs. Restriction sites are indicated.

For colony forming assays, transfected cells were plated in a 10 cm dish containing 10 mL of nonselective medium. Three days after transfection, 3 $\times$ 10⁵ cells were plated per 10 cm dish in -GHT medium (DMEM:F12 supplemented with 7.5% dialyzed fetal bovine serum [Sigma, St. Louis, MO], 6 mM L-glutamine, 1 mM asparagine) containing 10 nM methotrexate (MTX) (Lederle Laboratories, Pearl River, NY). Nine to eleven days later, the cells

Figure 4. Recombinant protein production from pools made with EASE and non-EASE vectors. Black bars represent specific productivity of pools generated using the non-EASE containing vector pGλFLT3L. Diagonal striped bars represent specific productivity of cell pools generated with the EASE containing vector, pG2.1FLT3L. FLT3L was measured using ELISA assays. Only one pool using pGλFLT3L grew at 200 nM MTX compared with four using pG2.1FLT3L. Error bars represent standard error of the mean.

were fixed (2% formaldehyde [Sigma], 0.2% glutaraldehyde [Sigma] in phosphate buffered saline) and then stained with 2% methylene blue. Colonies were counted, and the counts were normalized to the amount of DHFR encoding sequence used for each electroporation.

Amplification, specific productivity and plasmid stability

For experiments involving recombinant protein expression and amplification of DHFR, cells were recovered from electroporation for 3 days in nonselective medium, and then transfected cells were seeded at 1×10^6 cells per T75 flask in -GHT medium. They were allowed to grow to confluency and then seeded again at 1×10^6 cells per T75 flask in -GHT medium. After the second round of selection, they were seeded at 1×10^6 cells per T75 flask in -GHT medium supplemented with either 50 nM, 100 nM or 200 nM MTX and grown to confluency. They were grown a second time in 50, 100 or 200 nM MTX before specific production of recombinant protein was measured.

For production cultures and the stability study the amount of recombinant protein expressed was quantitated by determining the average microgram of protein produced per 10^6 cells per day (specific productivity). Protein titers were measured by ELISA (Engvall and Perlmann, 1972). Plasmid stability was investigated by comparing specific productivity of a recombinant protein expressed by cell pools cultured in -GHT media supplemented with 100 nM MTX. Specific productivity was measured every five passages. For the production cultures, cells were seeded at 2×10^6 cells mL^{-1} in T125 shake flasks. Cultures were then induced and protein production, cell viability and glucose consumption was monitored for 10 days at which time the cultures were terminated. All cultures were handled identically.

Southern hybridizations

High molecular weight genomic DNA was extracted using previously described methods (Mitchell et al., 1986). The DNA was quantitated with a fluorometer (Hoefer, San Francisco, CA), digested with appropriate enzymes and then fractionated on 1% tris-acetate buffered agarose gels as described (Sambrook et al., 1989). DNA fragments were separated using a continuous electrophoresis apparatus (Owl Scientific, Woburn, MA). The DNA was transferred to Zeta-Probe GT Genomic Tested Blotting Membrane (Bio-Rad, Hercules, CA) as recommended by the supplier. Prehybridization and hybridization to P^{32} (Amersham, Arlington Heights, IL) *flt3l* probes prepared using a Random Primed DNA Labeling Kit (Boehringer-Mannheim Biochemicals) were performed at 60 °C. The filters were exposed to Kodak XAR5 film for an appropriate time. The screens were scanned using a Personal Densitometer SI (Molecular Dynamics, Sunnyvale, CA) and analysis and quantitation were accomplished using ImageQuant software (Molecular Dynamics).

Statistical analysis

Analysis of variance of group means and linear regression was performed using the JMP Statistical Discovery Software (Cary, NC)

Results and discussion

Mapping of EASE activity with colony forming assays

In a previous study, it was shown that 2a5-3 λ and a derivative of 2a5-3 λ , pG5.7, (Figure 1 and Morris et al., 1997) could be used to develop stable cell pools expressing recombinant protein in a five- to seven-week time period. In the present study, a colony forming assay was used to map the activity in the 5.7 kb region of CHO DNA to a minimal element able to facilitate the production of CHO pools. A series of constructs were made containing various lengths of CHO DNA from 2a5 λ or control λ DNA placed 5' of the CMV promoter/enhancer of a plasmid expressing the amplifiable dominant selectable marker DHFR (Figure 1). Plasmid pG3.8 also served as a negative control plasmid as it contains a 3.8 kb segment of CHO DNA from 2a5 λ which previous studies had indicated was not necessary for EASE activity (Morris et al., 1997). In preliminary experiments, pG3.6 was shown to have the same activity as pG5.7 in the colony forming assays; thus, for the present study pG3.6 was used as the positive control. The transfected cells were plated in 10 nM MTX, scored for colony formation, and colony formation relative to pG3.6 was calculated. Mean relative colony formation for each vector was analyzed using analysis of variance (ANOVA) and differences in means were found to be highly significant ($p < .0001$). pG2.1 had an equivalent amount of colony forming activity compared to pG3.6 (Figure 2), and both had significantly more colony forming activity compared with the negative controls pG λ and pG3.8. Plasmid pG.2SE1.8 had more colony forming activity than the negative controls but less colony forming activity than pG3.6 and pG2.1 (Figure 2). From this study it appeared that pG2.1 contained most if not all of the EASE activity in pG3.6, so it was used for additional experiments.

Enhanced colony formation could reflect improved expression due to several mechanisms: 1) increased number of cells transfected (higher transfection efficiency), 2) higher copy number of transfected DNA at each integration site per cell or 3) increased transcription at each integration site. The experiments described below suggest that the EASE element facilitated colony formation by increasing gene copy number at a faster rate than control plasmids.

Expression of recombinant protein using EASE and non-EASE containing bicistronic vectors

To determine if the 2.1 kb EASE identified in the colony forming assays could be used in expression vectors to facilitate recombinant protein production, this element and the 2.0 λ control DNA were used in bicistronic vectors to express FLT3L, a soluble hematopoietic growth factor. A cDNA for FLT3L (Brasel et al., 1996) and the encephalomyocarditis virus internal ribosomal entry site (nucleotides 260-824) (Jang and Wimmer, 1990) were cloned into pG2.1 and pG λ to create pG2.1FLT3L and pG λ FLT3L (Figure 3), respectively. The plasmids were linearized using *FspI* for pG2.1FLT3L and *BamHI* for pG λ FLT3L. Four independent electroporations into DXB11 CHO cells were performed for each construct. Electroporated cells were grown to confluency in DHFR selective media and subsequently grown in 50, 100 or 200 nM MTX. Cells electroporated with pG2.1FLT3L grew in 100 nM MTX selective media significantly faster than cells electroporated with pG λ FLT3L (t-test with $p < 0.05$) (Table I). Only one pG λ FLT3L pool grew in the 200 nM MTX compared with four for pG2.1FLT3L, and it took about 5 weeks longer to reach harvest (Table I). There was no difference in the growth rate of cell pools in the 50 nM MTX supplemented media. The fact that the cell pools containing EASE vectors grew faster under higher selective pressure supports the hypothesis that the pG2.1FLT3L vector was able to amplify more rapidly compared with the control plasmid.

To determine if the production of recombinant protein in pools generated with the two plasmids was equivalent for each set of pools, specific production of FLT3L was measured for pools grown in different levels of MTX. At higher selective pressure, the pools from both vectors expressed increased amounts of protein indicating that both plasmids were able to increase expression due to MTX selection (Figure 4). Though the EASE containing pools had a higher average FLT3L expression at 100 and 200 nM MTX levels, upon statistical analysis there was no significant difference in the specific productivity between the pools generated with the different vectors at the same level of MTX.

The highest producing pools from selections done at 100 nM MTX using either pG2.1FLT3L (pG2.1FLT3L F5) or pG λ FLT3L (pG λ FLT3L B3) were tested for production of recombinant protein in a small scale shake flask production format. After cell

Table 1. Selection of cell pools in various levels of methotrexate requires less time with EASE vector

Vector	Days in		Total cells at confluency	
	100 nM MTX	200 nM MTX	100 nM MTX	200 nM MTX
pG2.1FLT3L	43 n=4	55±11 n=4	$1.49 \times 10^6 \pm 0.75$	$0.70 \times 10^6 \pm 0.42$
pGλFLT3L	54±7 n=4	93 n=1	$1.15 \times 10^6 \pm 0.74$	0.24×10^6

Only one pool using pGλFLT3L grew at 200 nm MTX compared with four using pG2.1FLT3L.

pools were adapted for growth in suspension for several passages, shake flasks were induced and cultured for 10 days. In a representative experiment, the EASE derived pG2.1FLT3L F5 pool maintained a higher specific productivity and titer throughout the course of the culture than the pGλFLT3LB3 pool (Figure 5). These data indicate that both vectors were able to generate pools that could be used to produce milligram amounts of soluble recombinant protein, but the pool generated with the EASE containing vector, pG2.1FLT3L F5, was available for production more quickly than the non-ease containing pool.

Prior to the production culture described above, the stability of recombinant protein expression was investigated for these two pools (Figure 6). Linear regression was used to determine if the protein production from the two pools was changing over the course of the study. The r^2 for the pG2.1FLT3L F5 and pGλFLT3LB3 were 0.19 and 0.45 respectively. The low r^2 values for both cell pools indicates that no statistically significant decrease in protein expression was detected. The higher r^2 value for pGλFLT3LB3 suggests there maybe a downward trend in protein expression for this pool. From a practical point of view, it is important to note that the production cultures (Figure 5) were performed approximately 10 passages subsequent to the stability study. This indicates that the stability of the pools was sufficient for generating milligram amounts of material under production conditions for an extended period.

Southern blot analysis of integration of plasmids in pools made with EASE and non-Ease vectors

To investigate the mechanism of action of the EASE containing vector at the molecular level, the integration pattern of the expression vectors for both sets of pools generated with 0 and 100 nM MTX

was compared using Southern blot analysis. High molecular weight genomic DNA from pools derived from pG2.1FLT3L transfected cells was digested with *HincII*, an enzyme that linearized the plasmid (Figure 3). The pGλFLT3L derived pool DNA was digested with *NotI* and *SphI*, enzymes that cut the plasmid once each to release a 6 kb fragment containing the FLT3L cDNA, the λ control DNA insert and the pGem1 plasmid backbone (Figure 3). A probe encoding FLT3L was used to detect integrated plasmid DNA. Pools created with pG2.1FLT3L showed two major bands at about 8 kb and >10 kb in pools selected at 0 and 100 nM MTX (Figure 7). The 8 kb band detected in the pG2.1FLT3L pools was the expected size for the unit length plasmid, suggesting that some of the EASE plasmid integrated and amplified in head to tail tandem arrays. The larger band could represent a head to head integration event which would produce a 13 kb band when cut with *HincII*. The relative intensity of this higher molecular weight band was increased relative to the 8 kb band in the 100 nM pools, indicating that this was the preferred amplification target. The pools generated with the EASE containing vector did show some bands whose sizes could not be explained by either head to head or head to tail integration events particularly after amplification, thus indicating the presence of some novel junctions. In contrast to the relatively simple patterns generated by pG2.1FLT3L transfected cells, the pools created with the pGλFLT3L plasmid showed complex banding patterns in pools at both MTX levels. Head to tail and head to head integration events would generate 6–7 kb bands which were not consistently detected at either MTX level. The amount of amplification for each set of pools was highly variable and no significant difference in the amount of amplification between pools was detected.

Figure 5. Production of FLT3L from pool pG2.1FLT3L F5 or pGλFLT3LB3 was compared. Each cell pool was suspension adapted and used in a small scale shake flask production experiment. FLT3L production was measured by ELISA. Each point represents the mean of duplicate samples.

Conclusions

In the current study it has been shown that a truncated 2.1 kb EASE element can be used in bicistronic vectors to create CHO cell pools expressing recombinant protein faster than a control vector which contained a 2.0 kb segment of λ DNA. The mechanism of action of the EASE element seems to be at the level of DNA integration and amplification. Southern blot analysis of DNA from cell pools generated with the EASE vector selected at low selective pressure (no MTX) had similar banding patterns. By contrast the pools selected with the control vector showed complex banding patterns with many interruptions to the expression plasmid. The amount of plasmid DNA initially integrated in the cell pools and amount of plasmid sequence in the DNA from the amplified pools were highly variable with no statistical difference between the sets of pools. In addition, there was no statistical difference in the protein expression level between pools. These data taken together indicate that the EASE element is causing an increased rate of amplification which would account for the shorter time line to get to an equivalent level of DNA copies and protein expression as the controls. By contrast, if the EASE were acting to enhance transcription, we would expect to see less integrated plasmid DNA in the EASE containing pools compared with the controls.

Other groups have described DNA sequences which promote gene amplification (Beitel et al., 1991; Holst et al., 1988; McArthur and Stanners, 1991), and their use in expression vectors (Hemann et al., 1994; Meyer et al., 1993; Weidle et al., 1988). The HSAG family of middle repetitive DNA sequences (Beitel et

al., 1991; McArthur and Stanners, 1991) is similar to EASE in that they increased colony formation in colony forming assays. The EASE sequence described here is distinct from the HSAG sequences since it does not correspond to a previously described middle repetitive sequence family and it is only able to increase colony formation 3 to 5 fold compared to the HSAG sequences which were able to increase colony formation by two orders of magnitude. In addition the HSAG DNA did not seem to change the integration pattern of plasmid DNA compared with controls as was the case for EASE. This effect could be due to the methods used for introducing DNA into the cells; for the HSAG studies calcium phosphate precipitation was used, and electroporation was used for the EASE studies reported here.

Weidle et al. (1988) have successfully used a sequence from murine rRNA, muARS4 in expression constructs containing a crippled promoter driving the selectable marker. These authors report that stable expression in individual clones was dependent on culturing cell lines in the presence of selective pressure and that the expression plasmid DNA appears to be maintained in the transformed cells both episomally as well as integrated in the chromosome. Cell lines cloned from pools made with EASE containing plasmids are stable when cultured for at least 70 generations without selective pressure (data not shown), indicating that EASE containing plasmids are stably maintained integrated in the chromosome.

An additional murine ribosomal element muNTS1 has been used for recombinant protein expression (Hemann et al., 1994; Meyer et al., 1993) and does not

Figure 6. Stability study of pools pG2.1FLT3L F5 and pGλFLT3LB3 grown in selective media supplemented with 100 nM MTX for 32 passages. Specific productivity was measured using ELISA.

require selective pressure to maintain stable expression (Meyer et al., 1993). It is difficult to determine the similarities and differences between the mechanism of action of the EASE, muARS4 and muNTS1 elements as they have been developed in different experimental systems. It appears that the muARS4 and muNTS1 elements increase the total amount of amplification at a given selective pressure whereas the EASE decreases the amount of time necessary to reach a set amount of amplification.

In summary, the EASE is able to improve protein expression most likely by increasing the rate of plasmid DNA amplification when cells are grown under increasing selective pressure. The EASE appears to be unique since its ability to promote amplification is moderate compared to the HSAG middle repetitive sequence family. In this report we have shown that the cell pools expressing recombinant protein can be generated with the EASE vectors without any cloning steps making them apparently easier to use than the muNTS1 vector which require cloning to select high expressing cell lines (Hemann et al., 1994; Meyer et al., 1993). EASE containing bicistronic vectors have been used to generate milligram amounts of protein

from serum free and serum containing cell pools for many proteins (Morris et al., 1997), using efficient two step selection protocols.

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Figure 7. Southern blot analysis of DNA isolated from cell pools generated with EASE and non-EASE containing plasmids. The amount of MTX used to select each pool is indicated above the lanes. Pools F1, F2, F4, F5 were generated using plasmid pG2.1FLT3L. Pools B2, B3, B4, B5 were generated using plasmid pG λ FLT3L. The DNA from pools generated with pG2.1FLT3L were digested with *HincII*. The DNA from pools generated with pG λ FLT3L were digested with *NotI* and *SphI* restriction enzymes. The locations of the molecular weight markers are indicated on the right. The panel on the right represents the autoradiograph image after 72 h. The panel on the left represents the autoradiograph image after 24 h.

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