An Internal Ribosome Entry Site (IRES) Mutant Library for Tuning Expression Level of Multiple Genes in Mammalian Cells

Esther Koh, Steven Ho, Mariati, Zhiwei Song, Xuezhi Bi, Muriel Bardor, Yuansheng Yang, Linda Hendershot

To cite this version:

Esther Koh, Steven Ho, Mariati, Zhiwei Song, Xuezhi Bi, et al.. An Internal Ribosome Entry Site (IRES) Mutant Library for Tuning Expression Level of Multiple Genes in Mammalian Cells. PLoS ONE, Public Library of Science, 2013, 8 (12), pp.e82100. 10.1371/journal.pone.0082100. hal-01843928

HAL Id: hal-01843928
https://hal-normandie-univ.archives-ouvertes.fr/hal-01843928
Submitted on 28 Jun 2019

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L’archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d’enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.
An Internal Ribosome Entry Site (IRES) Mutant Library for Tuning Expression Level of Multiple Genes in Mammalian Cells

Esther Y. C. Koh¹, Steven C. L. Ho¹, Mariati¹, Zhiwei Song¹, Xuezhi Bi¹, Muriel Bardor¹,², Yuansheng Yang¹,³*

¹Bioprocessing Technology Institute, Agency for Science, Technology and Research, Singapore, Singapore, ²Laboratoire Glyco-MEV (Glycobiologie et Matrice extracellulaire végétale), Université de Rouen, Mont-Saint-Aignan, France, ³School of Chemical and Biomedical Engineering, Nanyang Technological University, Singapore, Singapore

Abstract

A set of mutated Encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES) elements with varying strengths is generated by mutating the translation initiation codons of 10th, 11th, and 12th AUG to non-AUG triplets. They are able to control the relative expression of multiple genes over a wide range in mammalian cells in both transient and stable transfections. The relative strength of each IRES mutant remains similar in different mammalian cell lines and is not gene specific. The expressed proteins have correct molecular weights. Optimization of light chain over heavy chain expression by these IRES mutants enhances monoclonal antibody expression level and quality in stable transfections. Uses of this set of IRES mutants can be extended to other applications such as synthetic biology, investigating interactions between proteins and its complexes, cell engineering, multi-subunit protein production, gene therapy, and reprogramming of somatic cells into stem cells.

Introduction

Simultaneous expression of multiple genes in mammalian cells at finely controlled amounts or ratios is required for applications such as synthetic biology, investigating interactions between proteins and its complexes, cell engineering, multi-subunit protein production, gene therapy, and reprogramming of somatic cells into stem cells [1,2,3,4]. Three common strategies for controlling multiple gene expression in mammalian cells are (i) co-transfection of multiple vectors at different relative amounts [5,6,7], (ii) single vector containing multiple promoters or polyadenylation signals with different strengths [8,9,10], and (iii) insertion of splicing signals with varied splicing efficiencies between genes [11]. Co-transfection is an inaccurate approach as the relative amount of different genes incorporated varies from cell-to-cell due to variations in transfection efficiency [12,13]. Using a single vector with multiple promoters or polyadenylation signals ensures the introduction of several genes into each cell at identical amounts and provides accurate control of gene expression in transient transfections [9]. However, the expression ratio still varies between cells in a stably transfected cell pool [7,13,14] as the arrangement of multiple promoters in close proximity causes transcriptional interference, where the active expression of one gene suppresses expression of the other genes. The degree to which gene expression is suppressed depends on the integration site in the genome [15]. The use of splicing signals allows stricter control of relative gene expression as all genes are expressed in one transcript [11]. This method requires elimination of cryptic splicing sites in protein coding sequences to prevent incorrect RNA splicing.

Co-expression of multiple genes in one mRNA for strict control of relative gene expression can also be achieved by using either 2A elements or internal ribosome entry site (IRES). 2A linked genes are expressed in one single open reading frame (ORF) and “self-cleavage” occurs co-transcriptionally to give equal amounts of co-expressed proteins [16]. This method does not allow modulation of the expression ratio between the proteins of interest. Moreover, incomplete cleavage of 2A peptides often results in attachment of additional unwanted amino acids residues to proteins and formation of fusion proteins [17,18,19,20]. When IRES elements are included between multiple open reading frames (ORFs), the first ORF is translated by the canonical cap-dependent mechanism while the rest are translated through a cap-independent mechanism [21,22,23]. It has already been shown that IRES allows strict control of the relative gene expression in both transient and stable transfections [13,24,25]. In contrast to the 2A element, products generated using IRES do not form any undesirable modified or fusion proteins [17,20]. More importantly, as genes are translated independently, the relative expression of different genes can be adjusted by varying the strength of the IRES applied to each gene. Although expression can be altered by using different naturally available IRES, the range of expression levels obtained is narrow due to the limited range of IRES elements [24]. The range of
IRES activity can be widened by mutating its sequence [26,27]. An earlier study generated a set of eleven IRES mutants by error prone PCR which controlled gene expression level across a 20-fold range [28]. However, the strengths of these IRES mutants appear to be cell specific as four IRES mutants that exhibited up to 36% strength differences in HEK293T cells showed no apparent difference in CHO K1 cells.

Encephalomyocarditis virus (EMCV) IRES is the most widely used IRES element for multiple gene expression in mammalian cells because of its superior activity in different cell lines and ability to mediate accurate translation [26,29,30]. The region that contributes to efficient EMCV IRES translation contains twelve AUGs [31]. Translation initiation occurs primarily at the 11th AUG (AUG-11), partially at the 12th AUG (AUG-12), and almost none at the 10th AUG (AUG-10) [32,33]. In cap-dependent translation, ribosomes can recognize codons other than AUG and initiate translation with reduced efficiency and gene expression. These non-AUG codons in mammalian cells have a relative rank order of translation efficiency of AUG>CUG>GUG>ACG>G>AUG>UU>UGG [34,35,36]. If IRES-mediated cap-independent translation follows the same principle, generation of EMCV IRES mutants with AUG-10, AUG-11, and AUG-12 mutated to non-AUG triplets should provide a new tool for accurate control of the expression level of multiple genes in mammalian cells.

In this work, we generated a series of mutated EMCV IRES by either deletion or mutation of AUG-10, AUG-11, and AUG-12 in the wild type EMCV IRES (IRESwt). They were evaluated in different mammalian cell lines and their use was demonstrated for optimization of light chain (LC) to heavy chain (HC) expression ratios for enhancing monoclonal antibody (mAb) expression level and quality. Different ratios of LC over HC expression affect both mAb expression levels and quality [7,14,37]. The optimum LC:HC ratio for mAb production depends on the cell type used for expression, whether production is performed using transient or stable transfections, and possibly the mAb molecule itself [7,20]. Here, we demonstrated that using different IRES mutants to express LC and HC allowed the control of LC:HC ratios at a level most beneficial for mAb expression and quality. This set of IRES mutants can be also used in other applications such as synthetic biology, investigating interactions between proteins and its complexes, cell engineering, multi-subunit protein production, gene therapy, and reprogramming of somatic cells into stem cells. The crippled EMCV IRES mutants with reduced translation efficiencies would be particularly useful for expressing proteins at more biologically relevant levels in the cell, or for expressing proteins that are toxic to the cells if over expressed.

### Materials and Methods

**Cell Culture and Media**

Adherent CHO K1, HEK293, BHK, 3T3, and COS7 cells (ATCC, Manassas, VA) were grown in the Dulbecco’s modified Eagle’s medium (DMEM) + GlutaMax™ (Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (Sigma, St. Louis, MO) in a static humidified incubator with 5% CO₂ at 37°C. Suspension dihydrofolate reductase (DHFR)-deficient CHO DG44 cells (Life Technologies) were grown in a protein-free medium consisting of 50% HyQ PF (HyClone, Logan, UT) and 50% CD CHO (Life Technologies) supplemented with 1 g/L sodium carbonate (Sigma), 6 mM glutamine (Sigma), 0.1% Pluronic F-68 (Life Technologies), and 1% hypoxanthine and thymine (HT) (Life Technologies) in humidified Kühner shakers (Adolf Kühner AG, Birsfelden, Switzerland) with 8% CO₂ at 37°C.

**Generation of EMCV IRES Variants**

The IRESwt with sequence corresponding to the region from 260 to 848 in the EMCV-R genome (Genbank: M81861) was cloned from the pIRES2-DsRed vector (Clontech Laboratories, CA). It contained two mutations compared to the sequence deposited in the Genbank (G at 739 to A and insertion of one A after 769) and is less active than the wild-type version [26]. The mutated EMCV IRES variants were generated by either mutation of AUG-10, AUG-11, and AUG-12 individually or in combination to GUG, CUG, ACG, AUA, UUG, or deletion of AUG-11 and AUG-12 and surrounding sequence, or both (Table I). The mutations were obtained by using synthetic primers containing specified mutations during PCR amplification.

**Vector Construction**

The IRES-mediated dual-luciferase bicistronic vectors containing different IRES variants on the firefly luciferase (Fluc) gene were constructed by replacing the BGHpA-CMV’ region in a previously described vector [10] with specific IRES variants (Figure 1A). The IRES-mediated tricistronic vectors containing different IRES variants on LC and HC were constructed by

| Table I. Relative strength of IRES variants in expressing a gene. |
|---------------------------------------------------------------|
| **EMCV IRES 3’-end Sequence** | **Strength (%)** | **Variant ID** |
|--------------------------------|-----------------|---------------|
| AUGAUAAUAUUGGCCACAACCAUG | 100.00 ± 0.00   | IRESwt        |
| GUGUAUAUAUUGGCCACAACCAUG | 90.48 ± 0.12    | IRESv1        |
| AUGUAUAUAUUGGCCACAACCGUG | 83.59 ± 1.72    | IRESv3        |
| AUGUAUAUGUGGCCACACAACCGUG | 35.48 ± 3.50   | IRESv7        |
| AUGUAUAUGUGGCCACACAACCGUG | 67.42 ± 2.91    | IRESv4        |
| AUGUAUAUGUGGCCACACAACAGUG | 29.45 ± 1.86    | IRESv10       |
| AUGUAUAUGUGGCCACACAACAGUG | 0.98 ± 0.02     | IRESv19       |
| AUGUAUAUGUGGCCACACAACAGUG | 1.37 ± 0.02     | IRESv18       |
| AUGUAUAUCUGGCCACAACAGUG | 45.18 ± 2.12    | IRESv5        |
| AUGUAUAUAUGCCACAACCAUG | 39.91 ± 0.80    | IRESv6        |
| AUGUAUAUAUGCCACAACCAUG | 34.39 ± 0.72    | IRESv8        |
| AUGUAUAUAUGCCACAACCGUG | 33.25 ± 0.36    | IRESv9        |
| AUGUAUAUAUGCCACAACCCUG | 9.47 ± 1.90     | IRESv13       |
| AUGUAUAUAUGCCACAACCGUG | 13.58 ± 1.50    | IRESv12       |
| AUGUAUAUAUGCCACAACCGUG | 4.02 ± 0.87     | IRESv14       |
| AGCAUAUAUAUGGCCACAACCGUG | 3.23 ± 0.18    | IRESv15       |
| AUGUAUAUAUGGCCACAACACAU | 1.79 ± 0.09     | IRESv16       |
| AUGUAUAUAUGGCCACAACACAU | 1.67 ± 0.16     | IRESv17       |
| AUGUAUAUAUGGCCACAACACAU | 0.58 ± 0.09     | IRESv21       |
| AUGUAUAUAUGGCCACAACACAU | 0.81 ± 0.03     | IRESv20       |
| AUGUAUAUAUG | 86.42 ± 0.03    | IRESv2        |
| AUG | 24.35 ± 4.04    | IRESv11       |
| AUGUAUAUGUG | 0.34 ± 0.20    | IRESv24       |
| GUGGAUAUGUG | 0.46 ± 0.10    | IRESv23       |
| GUG | 0.57 ± 0.12     | IRESv22       |

doi:10.1371/journal.pone.0082100.001

PLOS ONE | www.plosone.org 2 December 2013 | Volume 8 | Issue 12 | e82100
replacing the IRESwt-LC and IRESwt-HC regions in a previously described tricistronic vector expressing anti-HER2 mAb [37] with IRESvn-LC and IRESvn-HC, respectively (Figure 1B). IRESvn-LC and IRESvn-HC were synthesized by overlapping PCR. A short DNA sequence that can form a hairpin structure (HP) and contains an upstream AUG that is out of frame with the coding sequence of DHFR was inserted 101 base pair downstream of the predicted human cytomegalovirus IE gene promoter (CMV) transcription start site (Figure 1B). The out-of-frame AUG is present in an optimal sequence context for efficient initiation of translation. Insertion of such a DNA sequence immediately in front of the DHFR start codon is expected to reduce DHFR expression by about 50-fold [38] and enhance the stringency of selection for high producers in stable transfections [13].

**Transient Transfection**

Transient transfections for evaluating the strength of IRES variants in different mammalian cell lines were carried out in 6-well tissue culture plate (NUNC™, Roskilde, Denmark) using Fugene 6 (Roche, Indianapolis, IN). 24 h prior to transfection, 2 mL of exponentially growing cells at a cell density of 3 × 10^5 cells/mL were seeded into each well of the 6-well plates. Transfection of cultures in each well with the appropriate dual-luciferase vector was done in duplicates using a recipe of 6 μL of Fugene 6:2 μg of DNA. At 48 h post-transfection, cells were detached with trypsin and analyzed for renilla luciferase (RenLuc) and Fluc activities using Dual-Glo Luciferase Assay system (Promega, Madison, WI) in white opaque 96-well assay plates (Corning) in quadruplicates. The transfections and luciferase assays were repeated one more time using independently prepared plasmids and cultures.

At 24 h post-transfection, they were collected by centrifuge at 3000 g for 30 min at 4°C. The supernatants were then collected and quantified for concentrations of LC and HC polypeptides using alkaline phosphatase-conjugated goat anti-human IgG (Fc specific) for HC detection and goat anti-human IgG (Fc specific) for LC detection, respectively. Both detection antibodies were purchased from Sigma-Aldrich. The intracellular LC and HC polypeptides were detected simultaneously using two antibodies, HRP conjugated goat anti-human IgG Kappa LC antibody and HRP conjugated goat anti-human IgG Fc antibody. 10 pg of product was loaded for gels (Life Technologies) for analysis of LC and HC polypeptides in cell lysates. For analysis of supernatant, the sample loaded into each lane of NuPAGE 4–12% Bis-Tris gels (Life Technologies) was done in duplicates using a recipe of 6 μL of Fugene 6:2 μg of DNA. The intracellular polypeptides of LC:HC ratios in stable transfection pools generated using tricistronic vectors containing different IRES variants on LC and HC were determined using ELISA as previously described [13]. 1 × 10^7 cells were collected from 125 mL shake flask at 30 min at 4°C. The supernatants were then collected and quantified for concentrations of LC and HC polypeptides using alkaline phosphatase-conjugated goat antihuman IgG (Fc specific) for HC detection and goat anti-human IgG (Fc specific) for LC detection, respectively. Both detection antibodies were purchased from Sigma-Aldrich. The intracellular LC:HC polypeptide ratio in each stable transfection pool was determined as the measured LC concentration divided by the HC concentration.

**Western Blotting Analyses of Cell lysates and Supernatant**

Western blotting analyses of the cell lysates and supernatant were carried out as described previously [37]. 10 μg of total proteins were loaded into each lane of NuPAGE 4–12% Bis-Tris gels (Life Technologies) for analysis of LC and HC polypeptides in the cell lysates. For analysis of supernatant, the sample loaded into each lane contained 1 ng of mAb as determined by ELISA using Fc-specific detection antibodies. 10 pg of product was loaded for the sample with LC:HC of 21.24 to prevent overexposure due to the high levels of accumulated LC2 dimers and LC monomers. The LC and HC polypeptides were detected simultaneously using two antibodies, HRP conjugated goat anti-human IgG Fc antibody and HRP conjugated goat anti-human IgG Kappa LC antibody. Both detection antibodies were purchased from Bethyl Laboratories (Montgomery, TX).

**Generation of Stably Transfected mAb Producing Cell Lines**

Suspension CHO DG44 cells were transfected with the tricistronic vectors containing different IRES variants on the LC and HC genes (Figure 1B) by using electroporation on a Nucleofector (Lonz, Cologne, Germany). In each transfection, 1 × 10^7 cells were transfected with 5 μg of linearized plasmids. The transfected cells were then resuspended in 2 mL of protein-free medium preloaded in 6-well suspension culture plates (NUNC™). At 24 h post-transfection, they were collected by centrifuge at 1000 rpm for 5 minutes, and then resuspended in 15 mL of protein-free medium without HT and then followed by gene amplification in protein-free medium containing stepwise increased concentrations of methotrexate (MTX) at 50 nM and 250 nM respectively. To determine the productivity of mAb in stable transfection pools, 25 mL of cultures at a cell density of 2 × 10^5 cells/mL were seeded into 125 mL shake flask. Cell density and viability were monitored using Cedex counter (Innovatis, Bielefeld, Germany) until viability dropped below 50%. Supernatant was collected at the end of culture and analyzed for mAb concentration using a nephelometric method on an IMMAGE 800 immunochromistry system (Beckman Coulter, Buckinghamshire, England).

**ELISA Analysis of Intracellular Polypeptides of LC:HC Ratios**

The intracellular polypeptides of LC:HC ratios in stable transfection pools generated using tricistronic vectors containing different IRES variants on LC and HC were determined using ELISA as previously described [13]. 1 × 10^7 cells were collected from 125 mL shake flask at 30 min at 4°C. The supernatants were then collected and quantified for concentrations of LC and HC polypeptides using alkaline phosphatase-conjugated goat anti-human IgG (Fc specific) for HC detection and goat anti-human IgG (Fc specific) for LC detection, respectively. Both detection antibodies were purchased from Sigma-Aldrich. The intracellular LC:HC polypeptide ratio in each stable transfection pool was determined as the measured LC concentration divided by the HC concentration.
mAb Purification and Aggregation analysis

mAb in the supernatant was purified using protein A column on a GE AKTA explorer 100 (GE Healthcare, Uppsala, Sweden). The aggregation of protein A purified mAb was determined using size exclusion chromatography (SEC) coupled to a UV-visible detector and a dynamic light scattering detector. The instruments setup, chromatography conditions, and data analysis were as previously described (Ho et al. 2012).

LC-MS/MS Analysis of Signal Peptide Cleavage

Protein A purified mAb in stable transfection pools at different LC:HC ratios were analyzed for the signal peptide cleavage sites using NanoLC-MS/MS as described previously [17].

Results

Strengths of EMCV IRES Variants in CHO K1 Cells

The strengths of IRES variants were determined using dual-luciferase bicistronic vectors (Figure 1A) in transient transfections. The constructs were generated such that the 12th start codon for the EMCV IRES was used as the Fluc start codon. It has been reported that the expression of the first cistron (cap-dependent translation) is not affected by the downstream IRES-driven cistron (cap-independent translation) [39,40]. Thus Rluc was used as an internal standard to normalize the transfection efficiency for accurate determination of Fluc expression to reflect the strength of IRES variants. However, we observed that both Rluc and Fluc readings varied drastically both across different IRES variants and between the two independent experiments (Figure 2A and 2B). Rluc reading variations should be due to variations in transfection efficiency and Fluc reading variations could be due to both varied transfection efficiency and different strengths of IRES variants. After normalization of the Fluc reading to both the Rluc reading and the IRESwt, the effect different IRES variants had on Fluc expression were clear and highly reproducible in the two experiments (Figure 2C). The average relative strength and standard deviation of each IRES variant determined from the two independent experiments were calculated and reported in Table I. These 24 IRES variants enabled expression of Fluc to be controlled over a 300-fold range with small intervals and were sorted according to their relative strengths in descending order with IRESv1 referring to the strongest variant and IRESv24 referring to the weakest variant (Figure 2 and Table I).

All IRES variants exhibited reduced Fluc expression as compared to the IRESwt (Table I). The magnitude of reduction varied depending on the position and the number of mutated AUGs. Mutation of AUG-10 and AUG-12 to GUG reduced IRES strength to 90.48% and 83.59%, respectively, while mutation of AUG-11 reduced the strength more significantly to 35.48%. These results are consistent with previous reports that translation by IRESwt is initiated primarily at AUG-11, partially at AUG-12, and very rarely at AUG-10 [32,33,41]. Mutation of two AUGs had combinatorial effect in reducing the strength of IRES. For instance, mutation of AUG-10 and AUG-12 alone to GUG reduced the strength to 67.42%. Similarly, mutation of both AUG-10 and AUG-11 reduced the strength to 29.45% and mutation of both AUG-11 and AUG-12 reduced the strength to 0.98%. Interestingly, mutation of all three AUGs to GUG reduced the strength to 1.37%, showing no significant difference compared to mutation of AUG-11 and AUG-12 together as analyzed by two-sample t-test.

The type of bases used for AUG mutations affected the strength of IRES differently. For example, mutation of AUG-11 to CUG

![Figure 2. Relative strength of EMCV IRES variants in CHO K1 cells in transient transfections.](https://example.com/figure2.png)
Transfection of each vector was done in duplicates and repeated a second time using independently prepared plasmids and cultures. (A) and (B) Measured Fluc and Rluc activities. (C) Ratios of luciferase strengths were calculated as the ratios of luciferase activities of Fluc to Rluc gene for each IRES variant normalized to the wild-type IRES (WT). Each individual bar represents the average and standard deviation of eight measurements from two transfections in one experiment. Black bars represent the results from experiment 1 and gray bars from experiment 2.

doi:10.1371/journal.pone.0082100.g002

was least effective in reducing strength, giving strength of 45.18%, followed by AUA (39.91%), GUG (35.48%), UUG (34.39%), and ACG (33.25%). Two-sample t-test indicated that the differences between CUG and GUG (p<0.1), CUG and UUG (p<0.1), CUG and ACG (p<0.1), AUA and UUG (p<0.1), and AUA and ACG (p<0.1) were statistically significant. The effect that the type of base had on IRES strength changed when both AUG-11 and AUG-12 or all three AUGs were mutated, giving an order of CUG>ACG>AUA>GUG>UUG with strengths covering a range from 13.30% to 0.30%. Consistent with the observation for GUG, mutating all three AUGs to CUG, AUA, UUG, or ACG did not exhibit significantly reduced strength compared to mutations of AUG-11 and AUG-12 to the corresponding triplets as analyzed by using two-sample t-test. Deleting either AUG-12 alone or both AUG-12 and AUG-11 together with surrounding sequences was also effective in reducing IRES strength, resulting in strengths of 86.42% and 24.35%, respectively. Further mutations combined with the deletions generated three IRES variants IRESv24, IRESv23, and IRESv22 with strengths reduced to 0.34%, 0.46%, and 0.57%.

**Evaluation of EMCV IRES Variants in Different Mammalian Cell Lines**

To evaluate the application of IRES variants in different mammalian cell lines, IRESwt and three representative IRES variants, IRESv3, IRESv10, and IRESv18 that exhibited significantly different strengths in CHO K1 cells were tested in another four cell lines. The five cell lines were chosen from a range of species and tissues: CHO K1 from Chinese hamster ovary, HEK293 from human embryonic kidney, BHK from baby hamster kidney, 3T3 from mouse embryo, and COS7 from African Green Monkey kidney. The first three cell lines have been used widely for production of recombinant proteins in industry and the last two have been used in fundamental biological studies. The three IRES variants exhibited similar relative strengths to the IRESwt in expressing a gene in all different cell lines. The IRESwt was the strongest in all cases, followed by IRESv3, IRESv10, and IRESv18 (Figure 3).

**Application of EMCV IRES Variants for Controlling LC over HC Expressions**

The application of EMCV IRES variants for controlling the expression level of multiple genes in stable transfections was demonstrated by expressing a multi-subunit protein, IgG mAb. An IgG molecule is composed of two identical LC and two identical HC polypeptides linked by disulfide bonds. We first constructed a control anti-HER2 mAb expressing tricistronic vector with both LC and HC under the control of IRESwt. The control vector with IRESwt applied on both LC and HC demonstrated by expressing a multi-subunit protein, IgG mAb. An IgG molecule is composed of two identical LC and two identical HC polypeptides linked by disulfide bonds. We first constructed a control anti-HER2 mAb expressing tricistronic vector with both LC and HC under the control of IRESwt. The control vector with IRESwt applied on both LC and HC was not visible due to low expression.

The impact of LC:HC ratio on mAb yield was analyzed by plotting the end-point titer in shake flask batch culture against the LC:HC ratios as determined by ELISA (Figure 5). The control vector containing the IRESwt which controlled LC:HC ratio at 1.04 gave the highest titer of 84.1 mg/L. Slightly increasing the ratio to 1.49 by decreasing HC expression did not change titer significantly, yielding 83.3 mg/L. Further increasing the LC:HC ratios to 2.60 and 21.24 by decreasing the HC expression dramatically reduced the mAb titers to 34.0 and 5.9 mg/L, respectively. In contrast, a slight decrease of LC:HC ratio to 0.88 by decreasing the LC expression resulted in a sharp decline of mAb titer to 36.8 mg/L. The titer dropped to 17.1 and 8.1 mg/L when LC:HC ratios decreased to 0.24 and 0.04 by further reducing LC expression.

The qualities of mAb produced in stable pools at different LC:HC ratios were first characterized by western blotting analysis of culture supernatant under non-reducing conditions (Figure 6). Supernatant containing equal amounts of mAb determined by
ELISA was loaded into each lane of a NuPAGE gel. Lesser amount of mAb was loaded for sample at the LC:HC ratio of 21.24 to avoid overexposure of LC fragments. Product from the control vector with the LC:HC ratio of 1.04 contained complete IgG monomers HC2LC2 and a slight band corresponding to HC2 dimers, suggesting inefficient usage of HC polypeptides in mAb synthesis. Increasing LC:HC ratios to 1.49 and 2.60 by reducing HC expression resulted in secretion of less HC2 dimers. Further increasing the ratio to 21.24 eliminated secretion of HC2 dimers but resulted in secretion of LC2 dimers and LC monomers besides the complete IgG monomers, suggesting inefficient usage of LC polypeptides in mAb synthesis. In contrast, decreasing LC:HC ratios to 0.88, 0.24, and 0.04 resulted in secretion of HC2 dimers together with the complete IgG monomer, suggesting inefficient usage of HC polypeptides in mAb synthesis. Western blotting analysis of reduced products in supernatant was next performed. LC and HC polypeptides expressed from all different IRES variants had similar sizes compared to standard LC and HC polypeptides. HC band at the LC:HC ratio of 21.24 was not observed due to lower amount of sample loading.

To obtain a more accurate protein characterization, protein A purified mAb in stable pools at different LC:HC ratios were
separated on SDS-PAGE under reducing conditions. The excised bands corresponding to LC and HC polypeptides were digested by trypsin and then analyzed on LC-MS/MS for determining the signal peptide cleavage sites. The N-terminal tryptic peptide sequences of both LC and HC were confirmed by MS/MS via de novo and database matching using PEAKS program. Detection of peptide DIQMTQSPSSLSASVGDR and EVQLVESGGGLVQPGGSLR indicated that signal peptides of LC and HC were both cleaved at correct sites. No miscleaved signal peptide was observed on LC and HC in stable pools at LC:HC ratios of 1.04, 1.49, 2.60, and 0.24. LC-MS/MS analyses of products in stable pools at LC:HC ratios of 2.14 and 0.04 were not performed because of insufficient product.

Finally, the impact of LC:HC ratio on mAb aggregation was analyzed by using SEC coupled to a dynamic light scattering detector and UV detector. Supernatant collected at the end of culture was purified by protein A affinity chromatography before SEC analysis. Analysis of products in stable pools at LC:HC ratios of 21.24 and 0.04 were not performed due to insufficient product. One representative UV chromatogram for the pool of LC:HC = 0.24 is shown in Figure 7A. The molecular weight of each peak was calculated based on hydrodynamic radius determined by light scattering (data not shown). Peaks with average molecular weight greater and lower than the complete IgG monomers were grouped as aggregates and incomplete mAb fragments, respectively. Relative mass amounts of aggregate, complete IgG monomer, and incomplete mAb fragment were quantified using the respective peak area under the UV chromatograms. Analysis was done for duplicate stable transfection pools. Average distributions of components at different LC:HC ratios were shown in Figure 7B. Product at LC:HC ratio of 1.04 contained 96.2% IgG monomers, 3.6% aggregates, and 0.2% fragments. Increasing the ratio to 1.49 and 2.60 resulted in increase of IgG monomers to more than 99.3% and decrease of both aggregates and fragments to less than 0.5%. In contrast, decreasing the LC:HC ratio to 0.88 resulted in decrease of IgG

Figure 7. Representative size exclusion chromatogram and distribution of complete IgG monomer, aggregate, and incomplete IgG fragments produced in stable transfection pools at different LC:HC ratios. Components within protein A purified supernatant collected at the end of culture were separated by size exclusion chromatography followed by the identification and quantification of species by light scattering and UV detection, respectively. Analysis was done for duplicate stable transfection pools. (A) Representative chromatogram obtained by UV detector for the pool of LC:HC = 0.24. Agg: Aggregates; IgG: complete IgG monomer; Frag: Incomplete IgG fragments. (B) Quantitative comparison of complete IgG monomer, aggregates, and incomplete IgG fragments for different LC:HC ratios. Each value in figure B represents the average of four measurements from two independent stable transfection pools.

doi:10.1371/journal.pone.0082100.g007

Table II. Comparison of non-AUG translation efficiency in cap-dependent and cap-independent translations.

| Start condon | Cap-dependent translation efficiency (%) | EMCV IRES-mediated cap-independent translation efficiency (%) |
|--------------|------------------------------------------|-------------------------------------------------------------|
|              | Mutation of 11th AUG | Mutation of 11th and 12th AUG | Mutation of 10th, 11th and 12th AUG |
| AUG          | 100                       | 100.00                                      | 100.00                                      |
| CUG          | 50                        | 45.18                                      | 9.47                                        |
| GUG          | 20                        | 35.48                                      | 0.98                                        |
| ACG          | 10                        | 33.25                                      | 4.02                                        |
| AUA          | 5                         | 39.91                                      | 1.79                                        |
| UUG          | ND                        | 34.39                                      | 0.58                                        |

*Cap-dependent translation efficiencies of non-AUG condons were determined in COS-1 cells in transient transfections [35].
doi:10.1371/journal.pone.0082100.t002
monomers to 88.1% and increase of aggregates to 10.1% and fragments to 1.0%, respectively. Further decreasing the ratio to 0.24 did not worsen aggregation but saw a sharp increase of fragments to 23.3% and decreasing IgG monomers to only 70.6%.

Discussion

Translation represents a critical step in regulating gene expression in mammalian cells. During cap-dependent translation, ribosomes can recognize non-AUG triplets as start codons and initiate translation with reduced efficiency. The magnitude of reduction varies depending on the type of non-AUG triplets and their sequence context [34,35,36]. EMCV mRNA translation is initiated at AUG-11 or AUG-12 and less frequently at AUG-10 on internal ribosome entry sites (IRES) by a cap-independent mechanism [32]. We hypothesized that non-AUG initiated translation efficiency would be affected in similar ways in both cap-dependent and cap-independent translation. Mutation of AUG to non-AUG codons would reduce IRES translation efficiency and provide for greater precision during control of gene expression. Consistent with reports in cap-dependent translation studies, IRES variants with different non-AUG codons exhibited different strengths. However, the order of translation efficiency exhibited by different non-AUG was not the same as that reported in a previous study (Table II) [35]. Although we observed that mutating AUG-11 to CUG resulted in the lowest drop in translation efficiency, IRES variants with GUG-11, ACG-11, AUG-11, AUA-11, and UUG-11 did not exhibited strength differences as significant as those reported. One possible explanation is that when AUG-11 was mutated, efficient translation could be initiated at AUG-10 and AUG-12 [32]. When either both AUG-11 and AUG-12 or all three AUGs were mutated, the order of non-AUG codons for translation efficiency is the same as that in cap-dependent translation except for GUG. Conflicting orders for non-AUG initiation efficiency had been reported in different cap-dependent translation studies [34,35,36]. The different order of efficiency could be due to differences in sequence context around the start codons.

It has been reported that EMCV IRES variants generated by mutations of the critical domains in IRESwt exhibited cell-sensitivity [28]. Four IRES mutants that exhibited up to 36% difference in strength in HEK293T cells showed no apparent difference in CHO K1 cells. One possible explanation is that the strengths of these IRES variants depend on the interaction between trans-acting protein factors, such as polypyrimidine tract binding protein and the critical protein binding domains in EMCV IRES [42]. The expression level of these protein factors in CHO K1 cells may be lower than in HEK293T cells, resulting in decreased sensitivity of IRES strength to mutations of the critical domains. In contrast, the strengths of our newly generated EMCV IRES variants were manipulated by mutation of translation initiation sites, which may result in changes in ribosome binding affinity to the translation initiation start codon. As the molecular mechanism of translation initiation is highly conserved in all mammalian cells, it is not surprising that our IRES variants had similar relative strength in different cell lines.

We successfully controlled the LC over HC expression across a wide range of values using our IRES variants in stable transfections (Figure 4). Independent alteration of LC and HC expression provided better understanding of the mAb synthesis kinetics. Consistent with previous reports that expression of extra LC is more efficient for mAb expression [7,43,44], we found that at a balanced LC:HC ratio of 1.04, the mAb synthesis was not efficient as indicated by the secretion of HC fragment and occurrence of aggregates although this ratio gave the highest mAb titer (Figure 5). mAb production levels did not drop with a slight reduction of HC polypeptides, increasing LC:HC ratio from 1.04 to 1.49, but both aggregation and secretion of incomplete mAb fragment were minimized (Figure 6 and 7). In contrast, reduction of LC polypeptides by a similar magnitude (from 1.04 to 0.88) resulted in a sharp decrease in mAb production, a dramatic increase in aggregate, and secretion of HC fragments. It is known that mAb folding and assembly is more efficient when having extra LC polypeptides [44]. A slight reduction of HC polypeptides reduced the availability of substrate for mAb synthesis but having extra LC polypeptides enhances mAb folding and assembly, as a result mAb production level was not changed. However, the enhanced mAb folding and assembly reduced aggregation and enhanced the efficiency of LC and HC polypeptides usage for mAb synthesis. Similar mechanisms where a balance between substrate availability and folding efficiency is needed may explain the sharp decline in mAb production when LC polypeptides were slightly reduced, which not only reduced the substrate availability but having extra HC polypeptides may also reduce mAb folding and assembly, thus resulting in a sharp decrease in mAb titer and increase in aggregate. Substantial decrease of LC or HC polypeptides both resulted in sharp drop of mAb titers, as the amounts of substrates for mAb synthesis have become limiting. Also, extreme deviations from the balanced LC:HC ratios resulted in inefficient use of either LC or HC polypeptides for mAb synthesis. As such, we propose an optimized vector for mAb expression should express both LC and HC genes at high level and control the LC expression in slight excess.

Acknowledgments

The authors would like to gratefully thank Kong-Meng Hoi and Cheng Theng Ng for excellent technical assistance in regards to the mAb purification and aggregation analysis experiments and Dr Sheng-Hao Chao for critical review of the manuscript.

Author Contributions

Conceived and designed the experiments: XB MB YY. Performed the experiments: EYCK SCLH M XB MB. Analyzed the data: XB MB YY. Contributed reagents/materials/analysis tools: ZS XB MB. Wrote the paper: SCLH ZS XB MB YY.

References

1. Bieniossek C, Imasaki T, Takaşli Y, Berger I (2012) MultiBac: expanding the research toolbox for multigenic complexes. Trends in Biochemical Sciences 37: 49–57.
2. Fussenegger M, Moser S, Bailey JE (1998) Regulated multicistronic expression technology for mammalian metabolic engineering. Cytotechnology 28: 111–125.
3. Križa A, Schmid K, Baumgartner N, Ziegler U, Berger I, et al. (2010) A plasmid-based baculovirus expression system for mammalian cells. Nature Communications 1.
4. Trowitzsch S, Klumpp M, Thoma R, Carralot JP, Berger I (2011) Light it up: Highly efficient multigene delivery in mammalian cells. Bioessays 33: 946–955.
5. Leitzen K, Knüttler MB, Haas IG (1997) Assembly of immunoglobulin light chains as a prerequisite for secretion - A model for oligomerization-dependent subunit folding. Journal Of Biological Chemistry 272: 3117–3123.
6. Lenny N, Green M (1991) Regulation Of Endoplasmic-Reticulum Stress Proteins In Cos Cells Transfected With Immunoglobulin-Mu Heavy-Chain Cistrons. Journal Of Biological Chemistry 266: 20532–20538.
7. Schlatter S, Stansfield SH, Dinis DM, Racher AF, Birch JR, et al. (2005) On the optimal ratio of heavy to light chain genes for efficient recombinant antibody production by CHO cells. Biotechnology Progress 21: 122–133.
26. Bochkov YA, Palmenberg AC (2006) Translational efficiency of EMCV IRES in
25. Liu XD, Constantinescu SN, Sun Y, Bogan JS, Hirsch D, et al. (2000)
24. Sasaki Y, Sone T, Yahata K, Kishine H, Hotta J, et al. (2008) Multi-gene
23. Hellen CUT, Sarnow P (2001) Internal ribosome entry sites in eukaryotic
22. Mountford PS, Smith AG (1995) Internal Ribosome Entry Sites And Dicistronic
21. Martinez-Salas E (1999) Internal ribosome entry site biology and its use in
20. Chan HY, Sivakamasundari V, Xing X, Kraus P, Yap SP, et al. (2011)
16. de Felipe P, Luke GA, Hughes LE, Gani D, Halpin C, et al. (2006) E unum
15. Eszterhas SK, Bouhassira EE, Martin DR, Fiering S (2002) Transcriptional
14. Lee CJ, Seth G, Tsukuda J, Hamilton RW (2009) A clone screening method for expressing CHO cells: Conditional gene expression at near physiological levels. Journal Of Biotechnology 118: 123–134.
13. Ho SCL, Bardor M, Feng HT, Mariati, Tong YW, et al. (2012) IRES-mediated Tricistronic vectors for enhancing generation of high monoclonal antibody expressing CHO cell lines. Journal Of Biotechnology 157: 130–139.
12. Chusainow J, Yang YS, Yeo JH, Toh PC, Awadi P, et al. (2009) A study of monoclonal antibody-producing CHO cell lines: what makes a stable high producer? Biotechnol Bioeng 102: 1192–1196.
11. Fallot S, Ben Naya R, Hiebolt C, Mondlon P, Lacazette E, et al. (2009) Alternative-splicing-based bicistronic vectors for ratio-controlled protein expression and application to recombinant antibody production. Nucleic Acids Research 37.
10. Yang YS, Mariati, Ho SCL, Yap MGS (2009) Mutated Polyadenylation Signals from a single promoter. Journal of Biotechnology 136: 103–112.
9. Yahata K, Kishine H, Sone T, Sasaki Y, Hotta J, et al. (2005) Multi-gene Gateway clone design for expression of multiple heterologous genes in living cells: Conditional gene expression at near physiological levels. Journal Of Biotechnology 118: 123–134.
8. Tornoe J, Kusk P, Johansen TK, Jensen PR (2002) Generation of a synthetic mammalian promoter library by modification of sequences spacing transcription factor binding sites. Gene 297: 21–32.
7. Rees S, Coote J, Stables J, Goodson S, Harris S, et al. (1996) Bicistronic vector for the creation of stable mammalian cell lines that predisposes all antibiotic-resistant cells to express recombinant protein. Biotechniques 20: 102–104, 106, 108–110.
6. Li JD, Zhang CC, Jostock T, Dubel S (2007) Analysis of IgG heavy chain to light chain ratio with mutant Encephalomyocarditis virus internal ribosome entry site. Protein Engineering Design & Selection 20: 491–496.
5. Borman AM, Bailly JL, Girard M, Kean KM (1995) Picornavirus internal ribosome entry segments: Comparison of translation efficiency and the requirements for optimal internal initiation of translation in vitro. Nucleic Acids Research 23: 3656–3663.
4. Borman AM, LeMercier P, Girard M, Kean KM (1997) Comparison of picornaviral IRES-driven internal initiation of translation in cultured cells of different origins. Nucleic Acids Research 25: 923–932.
3. Duke GM, Hoffman MA, Palmenberg AC (1992) Sequence and structural elements that contribute to efficient encephalomyocarditis virus-RNA translation. Journal of Virology 66: 1602–1609.
2. Davies MV, Kaufman RJ (1992) The Sequence Context Of The Initiation Codon In The Encephalomyocarditis Virus Virus Mediates Efficiency Of Internal Translation Initiation. Journal of Virology 66: 1924–1932.
1. Kaminski A, Belsham GJ, Jackson RJ (1994) Translation Of Encephalomyocarditis Virus-Rna - Parameters Influencing The Selection Of The Internal Initiation Site. Embo Journal 13: 1673–1681.
40. Hennecke M, Kwissa M, Metzger K, Oumard A, Kroger A, et al. (2001) Requirement for internal initiation of translation of cardiovirus RNAs is different origins. Nucleic Acids Research 25: 925–932.
39. Davies MV, Kaufman RJ (1992) Gateway clone design for expression of multiple heterologous genes in living cells: Conditional gene expression at near physiological levels. Journal Of Biotechnology 118: 123–134.
38. de Quinto SL, Martinez-Salas E (1998) Parameters in influencing translational efficiency, aggregation, glycosylation and conformation stability. Journal Of Biotechnology 165: 157–166.
37. Ho SCL, Koh EYC, van Beers M, Mueller M, Wan C, et al. (2013) Control of IgG LC:HC ratio in stably transfected CHO cells and study of the impact on expression, aggregation, glycosylation and conformation stability. Journal Of Biotechnology 165: 157–166.
36. Peabody DB (1989) Translation initiation at non-AUG triplets in mammalian cells. Journal Of Biological Chemistry 264: 5031–5035.
35. Peabody DS (1989) Translation initiation at non-AUG triplets in mammalian cells. Journal Of Biological Chemistry 264: 5031–5035.
34. Kozak M (1989) Context Effects And Inefficient Initiation At Non-Aug Codons In Mammalian Transgenes. Trends In Genetics 11: 179–184.
33. Kozak M (1989) Context Effects And Inefficient Initiation At Non-Aug Codons In Mammalian Transgenes. Trends In Genetics 11: 179–184.
32. Davies MV, Kaufman RJ (1992) Gateway clone design for expression of multiple heterologous genes in living cells: Conditional gene expression at near physiological levels. Journal Of Biotechnology 118: 123–134.
31. Duke GM, Hoffman MA, Palmenberg AC (1992) Sequence and structural elements that contribute to efficient encephalomyocarditis virus-RNA translation. Journal of Virology 66: 1924–1932.
30. Kaminski A, Belsham GJ, Jackson RJ (1994) Translation Of Encephalomyocarditis Virus-Rna - Parameters Influencing The Selection Of The Internal Initiation Site. Embo Journal 13: 1673–1681.
29. Peabody DB (1989) Translation initiation at non-AUG triplets in mammalian cells. Journal Of Biological Chemistry 264: 5031–5035.
28. Peabody DB (1989) Translation initiation at non-AUG triplets in mammalian cells. Journal Of Biological Chemistry 264: 5031–5035.
27. Rees S, Coote J, Stables J, Goodson S, Harris S, et al. (1996) Bicistronic vector for the creation of stable mammalian cell lines that predisposes all antibiotic-resistant cells to express recombinant protein. Biotechniques 20: 102–104, 106, 108–110.