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Codon optimization of bacterial luciferase (*lux*) for expression in mammalian cells

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Abstract Expression of the bacterial luciferase (*lux*) system in mammalian cells would culminate in a new generation of bioreporters for in vivo monitoring and diagnostics technology. Past efforts to express bacterial luciferase in mammalian cells have resulted in only modest gains due in part to low overall expression of the bacterial genes. To optimize expression, we have designed and synthesized codon-optimized versions of the *luxA* and *luxB* genes from *Photorhabdus luminsecens*. To evaluate these genes in vivo, stable HEK293 cell lines were created harboring wild type *luxA* and *luxB* (WTA/WTB), codon-optimized *luxA* and wild type *luxB* (COA/WTB), and codon-optimized versions of both *luxA* and *luxB* genes (COA/COB). Although mRNA levels within these clones remained approximately equal, LuxA protein levels increased significantly after codon optimization. On average, bioluminescence levels were increased by more than six-fold [5×10^5 vs 2.9×10^6 relative light units (RLU)/mg total protein] with the codon-optimized *luxA* and wild type *luxB*. Bioluminescence was further enhanced upon expression of both optimized genes (2.7×10^7 RLU/mg total protein). These results show promise toward the potential development of an autonomous light generating *lux* reporter system in mammalian cells

Keywords Bioluminescence · Bioreporter protein · Eukaryotic expression

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Introduction

Mammalian cell lines expressing reporter proteins have been widely used in both basic and applied research for the investigation of a variety of cellular functions. For example, reporter proteins have been valuable tools for promoter analysis [9, 21], identification of transcription factors [11, 19], discovery of genes as potential targets for disease [20] evaluation of cross talk mechanisms [15], and in vivo sensing of tumor and/or disease progression [4]. However, current mammalian bioreporter technology is limited because of its inability to function as stand-alone, real-time reporter in vivo. Current methodologies that use firefly luciferase (Luc) and green fluorescent protein (GFP) reporter systems in mammalian cells require lysis and substrate addition or exogenous excitation, respectively, to produce a measurable response. Consequently, these cells are not practical choices for use in continuous on-line monitoring devices. Bacterial luciferase (*lux*) is unique in that it is the only bioreporter system available that generates its own substrate, thus eliminating the need for cell destruction or exogenous substrate addition.

Unfortunately, to date, a *lux*-based mammalian cell bioreporter has not been developed, due in part to the low expression levels of the bacterial luciferase enzyme in mammalian cells. The bioluminescence levels obtained by cloning wild type *luxA* and *luxB* genes encoding the heterodimeric luciferase enzyme into various mammalian cell lines have not demonstrated adequate bioluminescence for the development of reliable mammalian biosensors [1, 5, 7, 8, 10, 12, 16, 17]. Based on this information, it was determined that the *lux* genes need further optimization to realize their full potential as mammalian reporter proteins.

Codon optimization is the term given to the synthetic creation of a gene sequence to possess the optimal codon usage patterns for a specific host organism to enhance expression. Several examples of successful codon optimization have recently been published [2, 3, 6, 22]. These

optimized proteins have been designed primarily for increased expression in mammalian hosts, as mammalian expression of foreign genes is often low [14]. In this research, the *luxA* and *luxB* genes from the bioluminescent bacterium, *Photorhabdus luminescens* were optimized at the codon level and evaluated for enhanced expression in human cell lines.

Materials and methods

Strain maintenance and growth

Escherichia coli cells were routinely grown in Luria Bertani (LB) broth containing the appropriate antibiotic selection with continuous shaking (200 rpm) at 37°C. Kanamycin and ampicillin were used at final concentrations of 50 and 100 μg/mL, respectively.

All cell culture reagents and media were obtained from Sigma-Aldrich (St. Louis, Mo.) unless otherwise stated. Mammalian cells were grown in complete growth medium containing 10% heat-inactivated horse serum, 0.01 mM non-essential amino acids and 0.1 mM sodium pyruvate in a Dubelco's minimal essential media base (DMEM). Cells were routinely grown at 37°C in a 5% CO_2 atmosphere to confluence and split every 3–4 days by trypsinization at a 1:4 ratio. Neomycin G418 was used for selection at final concentrations ranging from 450 to 650 μg/mL according to kill curve analysis for each batch of antibiotic.

Determining codon-optimized sequence of *P. luminescens luxA* and *luxB* genes

The codon ratios within the wild type *lux* genes were analyzed and compared to optimal codon usage patterns from highly expressed (top 10%) mammalian genes as determined by information tabulated in GenBank. The overall ratio for usage of each codon within the wild type genes was altered to more closely match mammalian codon usage. The codons were replaced in a random fashion within the wild type sequences (Fig. 1). The sequence was further analyzed for potential splice sites and other regulatory regions using NetGene2 (http://www.cbs.dtu.dk/index.html). All potential splice sites were removed by nucleotide substitutions that resulted in silent mutations (Fig. 1). The final codon-optimized sequence was compared to the wild type sequence using Genescan (http://genes.mit.edu).

Synthesis of codon-optimized *luxA* and *luxB* genes

Oligonucleotides that spanned the complete sequence for each gene were designed, each containing an 18–23 bp overlap on the 5′ and 3′ ends with the adjacent oligonucleotide (Table 1). The overlapping regions were designed with melting temperatures (Tm) between 53 and 56°C. Oligonucleotides were synthesized by Sigma Genosys (St. Louis, Mo.) and polyacrylamide gel (PAGE) purified to ensure full-length products. Four reactions were set up with four adjacent oligonucleotides in each reaction. The oligonucleotides were linked by PCR (20 cycles and 50°C annealing) by adding internal oligonucleotides at 0.25 pmol and the two outermost oligos at 25 pmol.

The resultant PCR products were gel purified using a Geneclean gel extraction kit (Bio101, Carlsbad, Calif.). The extracted products were quantified and placed into a second PCR reaction at equal molar concentrations (0.25 pmol). The two outermost (5′ and 3′) oligos were used as primers at a final concentration of 25 pmol. Products of the correct size were again gel purified and TA TOPO cloned into the pCR4 TOPO cloning vector (Invitrogen, Carlsbad, Calif.). Resultant colonies positive for insert were sequenced to ensure sequence integrity. To correct base substitution errors introduced through PCR, site directed mutagenesis was performed. Final codon-optimized *luxA* and *luxB* genes were termed COA (GenBank accession no. AY581314) and COB (GenBank accession no. AY581315), respectively (patent pending).

Construction of a bicistronic expression vector

To compare expression of the codon-optimized *luxA* and *luxB* genes with that of the wild type, the pIRES vector was used (Clontech, Palo Alto, Calif.). This expression vector contains two multi-cloning sites separated by an internal ribosomal entry site (IRES) from encephalomyocarditis virus (EMCV). A wild type *luxA* and *luxB* (pWTA-I-WTB) plasmid, a codon-optimized *luxA* and wild type *luxB* (pCOA-I-WTB) plasmid, and a codon-optimized *luxA* and codon-optimized *luxB* (pCOA-I-COB) plasmid were generated. The wild type *lux* genes were amplified from *P. luminescens* (M90093) and the codon-optimized genes were generated as described above.

Transfection of mammalian cells

Transfection of mammalian cell lines was performed in six-well poly-D-lysine-coated tissue culture plates (Fisher Scientific, Pittsburgh, Pa.). HEK293 cells were split from stock cultures and inoculated into each well at approximately 1×10^5 cells per well in complete growth medium and grown to 80–90% confluency. The medium was refreshed on the day of transfection. DNA for transfections was purified from 100 mL overnight *E. coli* cultures using the Wizard Purefection plasmid purification kit (Promega, Madison, Wis.). Plasmid DNA was linearized by single restriction digestion before transfection to increase proper integration. Transfections were performed using the Lipofectamine 2000 reagent (Invitrogen); 24 h post transfection, the complexes were removed and the medium replaced with fresh complete

growth medium supplemented with the appropriate antibiotic.

Selection of mammalian cell clones

Selective medium was refreshed every 3–4 days. Within 2 weeks all control wells were dead and the transfected cells formed small colonies on the plate surface. Colonies were separated from the remainder of the well by placing a sterile chamber around the cell mass and sealing it with silicon (Fisher Scientific). The medium was then removed and each colony trypsinized and transferred to individual tissue culture flasks. Each colony was given a clone number and expanded to individual cell lines that were split and maintained as described earlier with the addition of selective medium. Twenty cell lines were propagated in this manner for each plasmid tested.

Bioluminescence assays from mammalian cells

Total proteins were extracted and in vitro enzyme (bioluminescence) assays performed. First, the cells were trypsinized from the flask surface according to standard protocols and resuspended in sterile phosphate buffered saline (PBS). The cells were then washed twice in PBS

Fig. 1 **a** Alignment of wild type *luxA* (*WTA*) and codon-optimized *luxA* (*COA*). **b** Alignment of wild type *luxB* (*WTB*) and codon-optimized *luxB* (*COB*). Base changes are indicated in *red*. *Blue* annotation indicates potential donor splice sites. *Green* annotation indicates potential acceptor splice sites. *Pink* annotation indicates a high probability ($>95\%$) splice site

Table 1 Oligonucleotide primer sequences used to synthesize the codon-optimized *luxA* and *luxB* genes

Primer name	Sequence 5′ to 3′
luxA Gene	
COA1 (sense)	5′ CGAAACCATGAAGTTCGGCAACTTCCTGCTCACATATCAGCC TCCCCAGTTTTCCCAAACCGAGGTCATGAAGCGGCTGGTTA AGCTCGGCCGCATCTC C 3′
COA2 (antisense)	5′ AAGCAGCAGCGACATAAGGGTTACCAAGCAGGCCGAA CTCGGTGAAGTGGTGCTCCAGCAGCCACACGGTGTCGAAAC CGCACTCCTCGGAGATGCGGCCGAGCTTA 3′
COA3 (sense)	5′ CCCTTATGTCGCTGCTGCTTATCTGCTCGGCGCCAC CAAGAAACTGAACGTCGGCACTGCCGCTATCGTTCTC CCCACCGC CCATCCAGTCCGCCAGCTT 3′
COA4 (antisense)	5′ GAAGTCCTTGTTGTAAAGCCCGCGGCAGATGCCGAA CCGAAAGCGCCCCT TGGACATTTGATCCAGCAAGTTC ACGTCCTCAAGCTGGCGGACTGGATGG 3′
COA5 (sense)	5′ CGGGCTTTACAACAAGGACTTCCGCGTGTTCGGCA CCGACATGAACAACAGCCGCGCCCTGGCCGAGTGTT GGTACGGGCTGATCAAGAATGGCATGA 3′
COA6 (antisense)	5′ GAGCGCCACCTCTGCTGTAAGCGGCGGGGTTCACTT TGACTTTGTGGAACTTGATGTGCTCATTGTCGGCTTCC ATGTATCCCTCGGTCATGCCATTCTTGATCAGCC 3′
COA7 (sense)	5′ ACAGCAGAGGTGGCGCTCCTGTTTATGTGGTGGCTG AGTCAGCTAGTACCACTGAGTGGGCTGCTCAATTTGG CCTCCCTATGATCCTGTCCTGGATCATCAACAC 3′
COA8 (antisense)	5′ CAGGCAGTGGTCGATGTTATGAATGTCGTGCCCG TACTCTTGAGCCACTTCGTTGTAAAGCTCGAGCTGG GCCTTCTTCTCATTAGTG TTGATG ATCCAGGACAGG 3′
COA9 (sense)	5′ CATAACATCGACCACTGCCTGTCCTACATCACCTC CGTGGACCACGACTCCATCAAGGCCAAGGAGATTTG CCGGAAGTTTCTCGGGCATTGGTATGATAG 3′
COA10 (antisense)	5′ AACACGAAATCGCGCCACTGCCCCTTGTTGAAGTC GTAACCTCTGGTCTGGTCGGAGTCGTCAAAGATAGTG GTAGCATTCACG TAGCTAT CATACCAATGCCCGAG 3′
COA11 (sense)	5′ AGTGGCGCGATTTCGTGTTGAAAGGACACAAGG ATACTAACAGACGCATCGACTACAGCTACGAGATCAA TCCCGTGGGCACCCCTCAG GAGTGCATTGACATCATCC 3′
COA12 (antisense)	5′ ATGGAAGCGATGATCTCGTCCACGGTTCCGTTAGCCTCA AATCCACAACAGATGTTGGAGATTCCGGTAGCATCAA TGTCCTTTTGGATGATG TCAATGCACTCCTG 3′
COA13 (sense)	5′ GACGAGATCATCGCTTCCATGAAGCTCTTCCAGTCCGATGTCA TGCCATTCCTCAAGGAGAAGCAACGCAG CCTCCTGTACTAGGGATCC 3′
COA14 (antisense)	5′ GGATCCCTAGTACAGGAGGCTGC 3′
luxB Gene	
COB1 (sense)	5′ CGAAACCATGAAGTTCGGACTGTTCTTCCTTAACTTCATCA ACTCCACCACTGTGCAGGAGCAAAGCATCGTGCGCATGCAGGAGATCACCGAGTATGTGGAC3′
COB2 (antisense)	5′ CACAGTCAGAGGAGCGCCGACAACGCCATTGTCGGAAAA GTGGTTCTCGTACACCAGGATCTGCT CGAAGTTCAGCTTG TCCACATAC TCGGTGATCTCC 3′
COB3 (sense)	5′ GGCGCTCCTCTGACTGTGTCCGGCTTCCTGCTCGGCCTGACCGAGAAGATCAAAATTGGCTCCCTGAACCACATCAT CACCACTCATCATCCTGTCGCCATCGCT 3′
COB4 (antisense)	5′ GTGCATCTCGTCCTTCTTCTCGCAATCGCTGAACCCC AGGATGAATCTCCCCTCGCTCAGCTGATCCAGCAGGCA AGCCTCCTCAGCGATGGCGACAGGATG 3′
COB5 (sense)	5′ GAGAAGAAGGACGAGATGCACTTTTTCAACCGCCCTGT GGAATATCAGCAG CAACTGTTTGAAGAGTGCTACGAGAT CATTAACGACGCTCTGACCACCGGCTACTGC 3′
COB6 (antisense)	5′ AGCGGTGACATACTTCCGAGGGCCGCCTGGGGTGTAA GCGTGGGGGTTGACGGAGATTTTAGGGAAGCTGTAG AAGTCATTGTCGGGGTTGCAGTAGCCGGTGGTCAG 3′
COB7 (sense)	5′ TCGGAAGTATGTCACCGCTACCAGTCATCACATCGTGG AGTGGGCTGCCAAG AAAGGCATCCCTCTCATCTTTAAGT GGGATGACTCCAACGACGTGAGATACGAGTA 3′
COB8 (antisense)	5′ TAACCAGGATCATCAGCTGGTGGTCGATTTCGGACAG GTCAACGTCATATTTGTCAGCCACGGCCTTGTATCTC TCAGCGTACTCGTATCTCACGTCGTTGG 3′
COB9 (sense)	5′ CCAGCTGATGATCCTGGTTAACTACAACGAAGACAGC AACAAGGCTAAG CAGGAGACCCGCGCCTTCATTAGCGA CTACGTGCTTGAAATGCACCCTAAC 3′

COB10 (antisense)	5′ CCAGCTTAGCAGCAGTGATACACTCGGTGTAGTTTCCG ACAGCGTTCTCGGCGATGATTTCCTCAAGCTTGTTCTCGA AGTTCTCGTTAGGGTGCATTTCAAGCAC 3′
COB11 (sense)	5′ TGTATCACTGCTGCTAAGCTGGCCATCGAGAAGTGCGGT GCTAAGAGTGTCCTGCTGTCCTTTGAGCCAATGAATGAC CTGATGAGCCAAAAGAACGTCAT 3′
COB12 (antisense)	5′ GGATCCTTAGGTGTACTCCATGTGGTACTTCTTAATATTG TCGTCCACAATGTTGATGACGTTCTTTTGGCTCATCAG 3′

(Sigma-Aldrich). Cell pellets were resuspended in 1 mL 0.1 M potassium phosphate buffer pH 7.8 and disrupted by three consecutive cycles of freeze (30 s liquid N_2)-thaw (5 min at 37°C) extraction. Cell debris was pelleted by centrifugation (14,000 *g* for 5 min) and the supernatant was used in the bioluminescence assay. To determine light intensity, 500 μL protein extract was mixed with 0.1 mM NAD(P)H, 4 μM FMN, 0.2% (w/v) BSA, 0.002% (w/v) *n*-decanal and a flavin oxidoreductase enzyme (1 U) isolated from *Vibrio harveyi* (Roche, Indianapolis, Ind.). Bioluminescence was measured using an FB14 luminometer (Zylux, Pforzheim, Germany) at a 1 s integration and reported as relative light units (RLU).

Bioluminescence signals were normalized between samples and cell lines by dividing the RLU measurement by the amount of total protein and reporting the bioluminescence as RLU/mg total protein. Protein concentrations were determined using the Coomassie Plus protein assay (Bio-Rad, Hercules, Calif.).

RNA isolation and northern blotting

At passage six, post-transfection, selected cell line clones were expanded to 75 cm^2 culture flasks. When the cells became 80–95% confluent, they were trypsinized and transferred to 2.0 mL Sarstedt tubes (Fisher Scientific). Total RNA was then isolated from the cells using an RNeasy kit (Qiagen, Valencia, Calif.). To remove any contaminating DNA, the RNA was digested for 30 min with DNaseI (Promega) and cleaned via the RNeasy clean up protocol. Total RNA was then quantified by absorbance at 260/280 (Beckman Coulter, Fullerton, Calif.).

Total RNA (10 μg) samples were electrophoresed and transferred to a Biotrans nylon membrane (ICN, Irvine, Calif.) using a semi-dry electroblot transfer apparatus (CBS Scientific, San Francisco, Calif.).

Double stranded DNA probes were generated complementary to a 300 bp portion of the codon-optimized and wild type *luxA* genes using standard PCR protocols with the incorporation of a [^{32}P] labeled dCTP nucleotide. Each probe was purified by column purification (Stratagene, La Jolla, Calif.). The specific activity of each probe was measured by scintillation counting (Beckman Coulter). Double stranded probes were boiled for 10 min to denature the DNA and directly added (in equal amounts of specific activity) to each blot. Specific activity from each sample was measured using a STORM 840 phosphoanalyzer and the data analyzed using ImageQuant data analysis software (Molecular Dynamics, Piscataway, N.J.).

Protein isolation and western blotting

Protein extracts were obtained and quantified as described above for bioluminescence assays. Equal amounts (250 μg) of protein were loaded and run on

12% SDS-PAGE gels. The proteins were transferred by electroblotting to PDVF membrane (Bio-Rad) using a semi-dry electroblotter (CBS, Del Mar, Calif.). Blots were blocked overnight in 5% nonfat dry milk and hybridized with a polyclonal antibody raised against a 16 amino acid *luxA* polypeptide [′N′(EM)FDDSDQTRGYDFNKGC(EM)′C′; Genemed Synthesis, San Francisco, Calif.). Antibodies were diluted 1:1,000 in T-TBS and applied to the membrane at room temperature. Blots were then incubated with a Goat Anti-Rabbit second antibody that has been conjugated to alkaline phosphatase and developed according to the manufacturer's protocol (Bio-Rad).

Results

Determination of a codon-optimized sequence of *P. luminescens luxA* and *luxB* for expression in mammalian cells

Upon sequence comparison, it was determined that the codon usage patterns between *P. luminescens* and human genes were extremely different. Therefore, to create an optimized version of the *lux* genes, the codon ratios were altered to more closely follow codon usage patterns within the human genome. Once the codon-optimized sequence was finalized it was tested using the GENSCAN online algorithm that predicts protein expression levels of gene sequences in human cells by comparing the sequence to highly expressed genes within the matrix specified (http://genes.mit.edu). The results of this analysis were encouraging and predicted a significant increase in expression on both transcriptional and translational levels (Table 2). Further, although verification was not possible, GENSCAN predicted a cleavage of the first 20 amino acids of the wild type LuxA protein when expressed in mammalian cells (Table 2). This cleavage was eliminated in the codon-optimized sequence and a full-length product was predicted.

Construction of the codon-optimized *luxA* and *luxB* genes

To evaluate the potential impact of codon optimization on the expression of the bacterial luciferase genes in mammalian cells, codon-optimized versions of each gene were required. To generate functional genes, single stranded oligonucleotides were linked by a two-step PCR process as described in Materials and methods.

In vivo expression of the wild type versus codon-optimized *luxA* and *luxB* genes

Wild type and codon-optimized versions of *luxA* and *luxB* were cloned into the pIRES mammalian expression vector to allow bicistronic expression of both genes with only one selection marker. Twenty stable clones (HEK293 cells) were selected for each construct along with one negative vector control. Each clone was tested in vitro for bioluminescence upon the addition of *n*-decanal and $FMNH_2$. These data revealed that each clonal cell line varied in its bioluminescence level (data not shown). On average, bioluminescence from the codon-optimized clones was significantly higher ($P < 0.05$) than bioluminescence from clones harboring wild type *lux* genes (Fig. 2). Based on these data, the two or three clones producing the highest bioluminescence levels from each gene combination were chosen for further study. At passage six, each clone selected was expanded into triplicate 75 cm^2 tissue culture flasks. From these cells, total genomic DNA, total RNA and soluble proteins were extracted for further analysis. From these individual clones, bioluminescence values (RLU/μg total protein) were found to be greater than two orders of magnitude higher in cell lines harboring both codon-optimized *luxA* and *luxB* genes (COA/COB) over that of cell lines harboring the wild type genes (WTA/WTB) (Fig. 3).

Determination of *luxA* mRNA levels in HEK293 clones

Transcript levels were determined to be approximately equal, with the exception of the WTA/WTB1 clone, which had a lower amount of *luxA* transcript (Fig. 4). The vector (NC) control had little to no background hybridization (Fig. 4).

Table 2 GENSCAN transcription and translation prediction scores for expression of wild type (*wt*) and codon-optimized (*op*) *luxA* and *luxB* in a human host (http://genes.mit.edu). Score interpretation: 0–50 weak, 50–100 moderate, >100 strong

Gene	Type	Begin	End	Length	*I*[a]	*T*[b]	CodRg[c]	*P*[d]	Translated[e]
LuxA (wt)	1	61	1,083	1,023	45	42	791	0.7	67.01
LuxA (op)	1	1	1,083	1,083	66	42	1,910	0.88	181.78
LuxB (wt)	1	1	984	984	51	38	585	0.97	46.37
LuxB (op)	1	1	984	984	66	41	1,952	0.99	185.60

[a] Initiation signal
[b] Termination signal
[c] Coding region score
[d] Probability of an exon
[e] Exon score

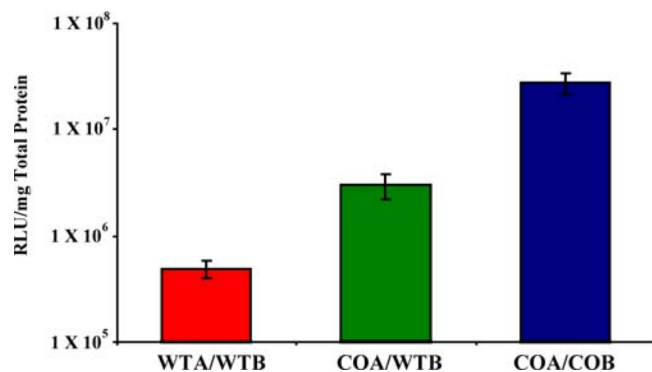

Fig. 2 Average bioluminescence from stably transfected HEK293 cell lines (20 clones tested for each clone type in triplicate)

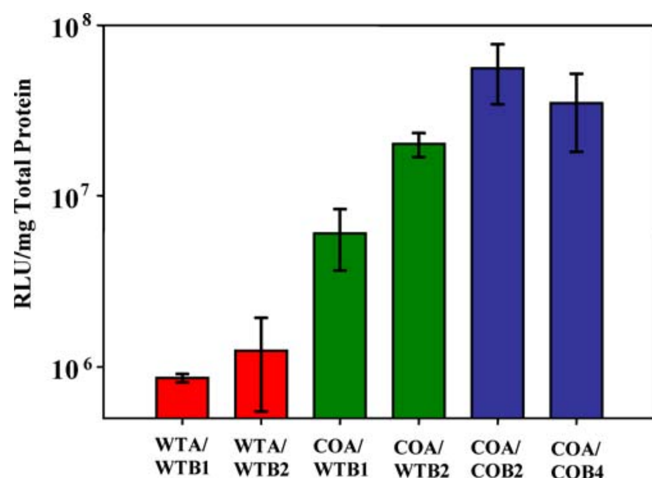

Fig. 3 Average bioluminescence from individual HEK293 clones producing the highest bioluminescence chosen from clones stably transfected with plasmids carrying either a wild type *luxA* and *luxB* (*WTA*/*WTB*), a codon-optimized *luxA* and wild type *luxB* (*COA*/*WTB*), or a codon-optimized *luxA* and codon-optimized *luxB* (*COA*/*COB*)

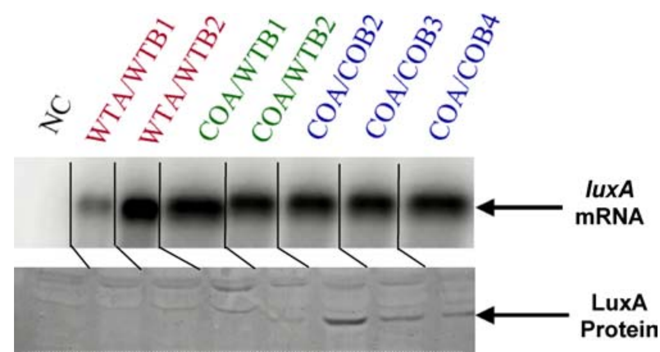

Fig. 4 Comparison of mRNA levels and protein levels in each of the stable HEK293 cell line clones. *Upper panel* Northern blot of total RNA (20 μg) from stably transfected HEK293 cells probed with ^{32}P-labeled complementary *luxA* probes. *Lower panel* Western blot of total soluble protein (250 μg) from stably transfected HEK293 cells immunoblotted with a polyclonal *luxA* antibody. *NC* Negative vector control

Determination of LuxA protein levels in HEK293 clones

LuxA protein was not detected from any of the wild type *luxA* and *luxB* clones, detected only at very low levels from codon-optimized *luxA* with wild type *luxB* clones, but readily detectable when both genes were codon-optimized (Fig. 4). This increase in LuxA protein concentration was observed despite the fact that the levels of *luxA* mRNA transcript were approximately equal for all of the clones tested (Fig. 4).

Discussion

Codon usage regulates gene expression at the level of translation, and codon usage patterns between species are not conserved [13]. This is especially true between genes derived from eukaryotes versus those from prokaryotes. Therefore, to efficiently express the bacterial *lux* genes in mammalian cells, the nucleotide sequence was altered in such a way as to create a ‘‘humanized’’ form of the gene without altering the amino acid sequence. This approach has been used previously to optimize the expression of both GFP and *Renilla* luciferase proteins for expression in mammalian cells [18, 22]. The design of this new sequence was carefully determined, removing all potential splice sites and most regulatory regions. According to the output from GENSCAN, the overall expression of the codon-optimized *lux* genes would be significantly improved versus that of the wild type. The increase in expression was predicted to be caused by an increase in both transcriptional and translational efficiency. Furthermore, it was predicted that the first 60 bases (20 amino acids) of the wild type *luxA* gene would be completely eliminated when expressed in mammalian cells. Considering that this region of the LuxA protein holds most of the catalytic properties (active site) for the bacterial luciferase enzyme, this would be devastating for the activity of the expressed protein. If this were the case, the low expression levels observed for the wild type genes may be explained in part by formation of a nonfunctional protein.

To test the expression of the codon-optimized genes, modified versions were required. However, because the necessary changes were too numerous to achieve by site directed mutagenesis alone, a complete in vitro gene synthesis protocol was pursued. The original plan was to amplify all of the oligonucleotides together in one PCR reaction according to methods described by Prodromou and Pearl [18]. However, because of the large size of the *lux* genes (approximately 1,000 bp each), this was not possible. As an alternative, each gene was synthesized in parts and subsequently linked by a second round of PCR.

Once constructed, wild type and codon-optimized genes were cloned into an IRES expression vector allowing bicistronic expression of both genes in mammalian cells. This expression format provides the most

natural platform for these genes, most closely mimicking the polycistronic form found in the bacterial operon. Bioluminescence increased significantly in the order WTA/WTB < COA/WTB < COA/COB. These data indicated that codon optimization had made a significant impact on the potential bioluminescence levels obtained from mammalian cells. To analyze this data further, the two or three brightest clones were chosen for further study.

Foreign gene integration in mammalian cells is a random event; therefore it is possible for more than one insertion of the construct to occur during each transfection. Since integration is fairly inefficient, the copy number per cell is generally very low. However, because of this possibility, the copy number of the inserted genes was determined. All of the cell lines tested had either one or two copies of the gene inserted with the exception of the COA/COB3 clone, which had three (data not shown). To simplify further measurements, this clone was disregarded for further bioluminescence comparisons. Nevertheless, it should be noted that increased copy number did not correlate with increased expression levels.

Transcript levels from the clones were approximately equal, with the exception of the WTA/WTB1 clone, which produced significantly lower amounts of *luxA* mRNA. Since each construct contained the same promoter (ECMV) element and initiation signals, it was expected that each clone would have approximately equal amounts of transcript for the introduced genes. However, cis acting regulatory elements could potentially interfere with transcription initiation and overall transcript levels in vivo. These types of interferences would vary based on where the genes were integrated within the chromosome. Therefore, the position effect of various clones could explain the lower amount of transcript detected with the WTA/WTB1 clone. Other factors that can potentially impact the amount of RNA transcript would result from increased RNA degradation of certain mRNA sequences that can occur in certain cases. This type of RNA instability would be less likely after codon optimization because of the removal of several AU-rich target degradation regions. However, because the lower amount of transcript was not seen in both the WTA/WTB clones tested, this scenario is unlikely.

The LuxA protein was not detectable from WTA/WTB clones and only faintly visible in the COA/WTB clones in Western blot analysis. However, large amounts of LuxA protein were detected from the COA/COB clones, which harbored a construct carrying codon-optimized versions of both genes. This finding was intriguing and unexpected. Since the only available antibody was raised against a polypeptide of LuxA, it was expected that the constructs harboring the COA/WTB and COA/COB would produce equal amounts of LuxA protein. However, this was not the case, indicating that the codon optimization of both genes might confer stability on the heterodimeric complex that makes up the luciferase enzyme. The increased stability of the complex may have allowed detection of the proteins in the Western blot while the other construct was not detected.

Perhaps the most valuable measurement to determine if codon optimization was a success is the amount of enzyme activity that could be obtained from each construct. Bioluminescence levels were evaluated on whole cell extracts upon addition of *n*-decanal and $FMNH_2$. Average bioluminescence values were found to be greater than two orders of magnitude higher in cell lines harboring both a codon-optimized *luxA* and *luxB* (COA/COB) over that of cell lines harboring wild type genes (WTA/WTB). While bioluminescence levels were significantly higher in clones expressing COA and WTB versus WTA and WTB, optimal bioluminescence was obtained from clones harboring optimized versions of both genes. These data further support the hypothesis of formation of a stable heterodimeric protein. Based on these data it was determined that codon optimization had a significant effect on the protein expression in HEK293 cells.

In conclusion, codon optimization of the *luxA* and *luxB* genes was successful in increasing the overall expression levels of the individual proteins in mammalian cells. This increase in protein quantity resulted in a significant increase in bioluminescence from mammalian cell lines harboring these constructs. Furthermore, the bioluminescence levels from codon-optimized *luxA* and *luxB* provided the high levels of bioluminescence needed for the development of reliable *lux*-based reporter constructs for analyte sensing in mammalian cells. Experiments are currently underway to express the full *luxCDABE* operon to generate the first fully autonomous mammalian bioreporter.

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