



## The level of synthesis and secretion of *Gaussia princeps* luciferase in transfected CHO cells is heavily dependent on the choice of signal peptide

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### Abstract

There is a great demand for the improvement of mammalian cell production systems such that they can compete economically with their prokaryotic counterparts. Of a number of parameters that need to be explored to accomplish this we have tested the effects of different signal peptides on the synthesis and secretion of *Gaussia princeps* luciferase in mammalian cells. A series of plasmids were transfected into CHO cells where the coding region for the marine luciferase was fused to the signal peptide coding regions derived from different sources. Both cell extracts and medium samples were analysed for luciferase activity. When the native *Gaussia* luciferase signal sequence in the vector was substituted by that from human interleukin-2 or albumin then the amount of active recombinant protein produced was substantially reduced, both in transiently and stably transfected cells. Western blotting showed that enzyme activity and protein levels mirrored one another. The major decrease in luciferase activity was shown not to be a result of decreased mRNA levels, indicating the involvement of a post-transcriptional event. When the coding region of human endostatin was fused to that of the *Gaussia* luciferase signal peptide then an elevated level of secreted endostatin was observed compared to when that of the albumin signal peptide was used. Stable transfection of HepG2 cells with the different signal peptide constructs gave essentially the same results as seen in CHO cells. The overall results indicate that the choice of signal peptide can be imperative to ensure an optimal synthesis and secretion of a recombinant protein in a mammalian cell culture system. © 2007 Elsevier B.V. All rights reserved.

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## 1. Introduction

Although prokaryotic cells grow more rapidly, are cheaper to maintain and are easier to handle in the laboratory than mammalian cell cultures, they have some important disadvantages when it comes to producing mammalian proteins. A major problem is that the prokaryotic cell is deficient with respect to machinery for post-translational modification of the synthesised protein e.g. glycosylation. Bacteria also lack appropriate chaperones, and there is, therefore, a certain risk of misfolding of the protein. These two factors may cause a recombinant protein, produced in a bacterial expression system, to differ severely from the native protein, both when it comes to biological activity and immunogenicity. Another difficulty is that of solubility of the recombinant protein since it may be incorporated into inclusion bodies, which would complicate its purification. In addition there is a problem if the recombinant protein is to be used for therapeutic purposes in humans, in view of the fact that many bacteria have a high endotoxin content that may be pathogenic in nature. For patients, especially those to be subjected to long term treatment with repeated injections of a recombinant protein, there is a certain danger of transmission of toxins and infectious particles. Based on the above it is evident that considerable effort is necessary to bring mammalian systems to a level that will make them economically competitive with their prokaryotic counterparts.

A lot of work concerning the optimisation of the production of a recombinant protein by a transfected mammalian cell line has concentrated on modulating parameters affecting cell growth such as pH, CO<sub>2</sub>, ionic strength, and cell density (Wurm, 2004). Genetic engineering approaches have predominantly focused on increasing protein production by raising the level of transcription through e.g. the incorporation of a strong promoter region, an activator and/or enhancer (Darzacq et al., 2005; Fiering et al., 2000; Lee and Young, 2000; Verrijzer et al., 1995). A novel and very different approach would be to identify specific signal peptides mediating a high level of protein production. This has not yet been extensively explored in mammalian host systems, but is addressed in the present study.

As a reporter protein luciferase from the marine copepod *Gaussia princeps* was chosen because of the sensitivity of the assay system. In nature, after the

luciferase is synthesised by the copepod, it is packed into secretory vesicles for storage. The enzyme is released in bulk into the sea water along with its substrate following an appropriate stimulus such as pressure waves generated by a potential predator. The flash of light produced confuses the predator enabling the copepod to escape. Signal peptides from human interleukin-2 (IL-2) and human albumin were adopted as alternatives to the *Gaussia* luciferase signal peptide because both proteins are known to be secreted with great efficiency in nature. Another reason was that they exhibit different patterns of secretion. Albumin is constitutively expressed and its secretion occurs as a constant flow into the blood from hepatic cells to replace that removed from circulation. Interleukin-2, on the other hand, is produced and secreted in a “burst-like” manner upon appropriate stimulation of leukocytes. A further motivation for choosing the albumin signal peptide was the fact that as described by Partridge et al. (1999) its coding region was known not to affect mRNA stability when present in a chimaeric construct.

To our knowledge this is the first study reporting significant differences in synthesis/secretion of a recombinant protein in mammalian cells due to substitution of the signal peptide. The overall goal being to optimise vectors to be used for high-yield production of medically important proteins such as monoclonal antibodies.

## 2. Materials and methods

### 2.1. Vector construction

All constructs in this study were made based on the pTRE2hyg expression vector (Clontech Laboratories) and using a seamless cloning strategy (Chen et al., 2000). This is a PCR-based restriction site-free cloning method, used here to assemble the “secretion cassettes” containing selected untranslated regions (UTRs) and coding regions fused without any linker sequences. The cassettes were introduced into pTRE2hyg at the multiple cloning site and the constructs termed p followed by a four-letter code. The first, third and fourth letters refer to the 5'UTR, the coding region and the 3'UTR of *Gaussia* luciferase cDNA (GenBank accession no. AY015993), respectively. The second letter refers to

the source of the signal peptide, where G stands for *Gaussia* luciferase, I for human interleukin-2 (GenBank accession no. NM\_000586), and A for human albumin (GenBank accession no. V00494). The “\*” sign indicates codon optimisation for mammalian gene expression (Tannous et al., 2005). The following constructs were prepared: pGGGG, pGGG\*G, pGG\*G\*G, pGIG\*G, pGAG\*G.

## 2.2. Cell line and cell culture conditions

Cultures of CHO AA8 Tet-Off or CHO K1 Tet-On cells (Clontech Laboratories) were grown in monolayer in DMEM or  $\alpha$ MEM growth medium, respectively, supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin (all from Sigma–Aldrich). HepG2 cells were cultured in MEM supplemented with 1% non-essential amino acids (100 $\times$  ready to use solution), 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin (all from Sigma–Aldrich). The cells were incubated in a humidified atmosphere of 5% CO<sub>2</sub> at 37 °C. The seeding density was approximately  $2.0 \times 10^4$  cells/cm<sup>2</sup>, allowing the cells to reach confluency within 2–3 days. Cell cultures were split by trypsination and re-seeding. Cells were counted using the Nucleocounter system from Chemometec according to the manufacturer’s instructions.

## 2.3. Transfection of cells and selection for stable cell populations

Both CHO AA8 Tet-Off and CHO K1 Tet-On cells were transfected using Lipofectamine 2000 reagent (Invitrogen), whereas for the HepG2 cells, FuGene reagent (Roche) was used. The transfection was performed according to the manufacturer’s instructions. Optimal transfection efficiency for Lipofectamine 2000 was achieved using a mix of 4  $\mu$ g DNA and 10  $\mu$ l Lipofectamine 2000 reagent in 2 ml growth medium containing  $6.0 \times 10^5$  cells at 90% confluency. For FuGene, best results were obtained using 2  $\mu$ g of DNA and 2  $\mu$ l FuGene reagent added to  $1.0 \times 10^6$  cells in 2 ml growth medium. CHO AA8 Tet-Off cells were used for transient transfections and were harvested 24 h after transfection (not including the 6 h incubation period before medium shift).

To obtain stable populations containing the plasmid construct with which they had been transfected, CHO K1 Tet-On and HepG2 cells were cultivated without selection agent for 24 h after transfection. For the next 20 days cells were cultivated in a medium containing 400  $\mu$ g/ml hygromycin. After this period of selection, the amount of hygromycin in the medium was reduced to 200  $\mu$ g/ml and cultivation was continued for a further 10 days. Recombinant protein expression was then induced by addition of doxycycline to the growth medium to a final concentration of 1  $\mu$ g/ml. Cells were harvested after cultivation for a further 24 h.

## 2.4. Harvesting samples

Cells transfected with appropriate constructs or control vector were seeded in 6-well plates, each well containing 2 ml of fresh medium, and harvested 24 h post-transfection/induction. Medium samples were collected from the same wells. To avoid interference from detached cells, all medium samples were centrifuged at 425 rcf for 10 min at 4 °C, and the supernatant fluids were transferred to fresh tubes. Cells were scraped off the growth surface in 1 $\times$  PBS, counted and extracts were prepared by addition of lysis buffer (10 mM Tris–HCl, pH 7.4, 10 mM NaCl, 1.5 mM MgCl<sub>2</sub> and 0.5% NP-40). After incubation for 5 min at room temperature cell debris was removed by centrifugation at 10,000 rcf for 10 min at 4 °C. Supernatant fluids (cell extract), together with the medium samples, were assayed for recombinant protein.

## 2.5. Isolation of total RNA and Northern blot analysis

RNA was purified from stable populations of cells by Trizol (Invitrogen) extraction according to the manufacturer’s instructions. Ten micrograms total RNA in buffer was then added to dH<sub>2</sub>O to a final volume of 8  $\mu$ l. After the addition of 3.5  $\mu$ l formaldehyde, 10  $\mu$ l formamide and 2  $\mu$ l 5 $\times$ MOPS, incubation was performed at 65 °C for 12 min prior to electrophoresis. RNA was transferred to a nylon membrane by capillary blotting and fixed by exposure to a cross-linker/UV lamp. Probes specific for the coding regions of codon-optimised *Gaussia* luciferase and of GAPDH were generated and 50 ng of each probe was

radioactively labelled, using the PrimeIt RmT Random Primer Labelling Kit (Stratagene) according to the manufacturer's instructions, with 50 mCi [ $\alpha^{32}\text{P}$ ]dCTP (3000 Ci/mmol; Amersham Biosciences). Hybridisation was performed as previously described (Hovland et al., 1995).

### 2.6. Luciferase activity assay

Luciferase activity was measured as Relative Light Units (RLUs) emitted when the sample was mixed with 150  $\mu\text{l}$  coelentraine solution (Prolume) of  $A_{267} = 0.400$ , in a Lucy 1 luminometer (Anthos Labtec Instruments). The raw data obtained from the luminometer was corrected for dilutions made and cell number.

### 2.7. Immunoblotting

Samples from CHO K1 Tet-On cells stably transfected with various constructs were subjected to immunoblot analysis using either polyclonal rabbit anti-*Gaussia* luciferase antibody (generous gift from Bruce Bryan, Prolume) or polyclonal rabbit anti-human endostatin antibody (CYT Immune Sciences). Cells were seeded in six-well plates and samples harvested 24 h post-induction and prepared as described in Section 2.4. Protein concentrations in cell extracts were estimated using the Bradford protein assay (Bio-Rad Laboratories) and medium samples adjusted accordingly. Proteins were separated by electrophoresis on SDS-polyacrylamide gels, proteins transferred to nitrocellulose membranes and blotted with either rabbit anti-luciferase antibody (1:250) or rabbit anti-endostatin antibody (1:1000). Blots were developed with HRP-conjugated goat anti-rabbit antibody (1:2000) (Biorad) using the ECL system (Amersham Biosciences) according to the manufacturer's instructions.

### 2.8. Lactate dehydrogenase activity assay

Lactate dehydrogenase activity (LDH) was determined by mixing 100  $\mu\text{l}$  3 mM Na pyruvate, 100  $\mu\text{l}$  0,3 M phosphate buffer (pH 7.0) and 100  $\mu\text{l}$  of NADH solution (1 mg/ml) in a cuvette, with 650  $\mu\text{l}$  dH<sub>2</sub>O. After addition of 50  $\mu\text{l}$  of sample the LDH activity was measured as the decrease in  $A_{340}$  during a period of

3 min. One unit of activity corresponds to a decrease in  $A_{340}$  of  $0.01 \text{ min}^{-1}$ .

## 3. Results

### 3.1. Testing of leakage from transfected cells by LDH activity measurement

Before performing studies on the secretion of luciferase into the medium, it was necessary to establish whether or not leakage of cytoplasmic components may have occurred through the plasma membrane caused by damage due to the use of transfection reagent. This was important to assess since leakage could possibly be mistaken for secretion of the recombinant protein. The level of leakage from the cells was monitored by measuring the activity of LDH, a cytosolic marker enzyme, in the culture medium.

Lactate dehydrogenase activity was measured in medium samples obtained from cells transfected with pGGGG and the control vector pTRE2hyg. In addition, LDH was measured in a sample of fresh growth medium in order to quantify the background caused by enzyme activity present in the FBS.

The results (Fig. 1) indicated that leakage did occur in cultures of transiently transfected cells, whereas for stable cell populations no leakage was seen. These findings, in addition to the fact that cells transfected with

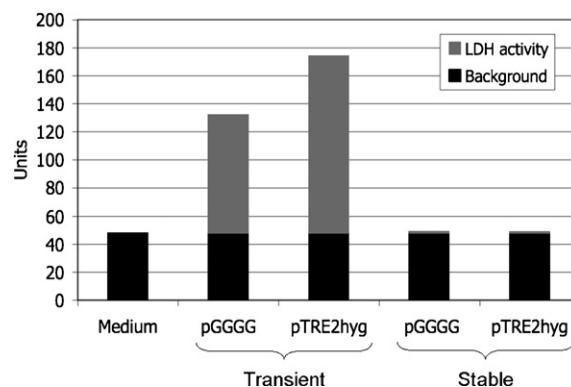


Fig. 1. Leakage of LDH from transfected CHO cells. Total LDH activity in medium taken from cells both transiently and stably transfected with pGGGG and control vector pTRE2hyg (grey bars). Background activity found in medium samples caused by LDH present in the FBS is shown (black bars).

the control vector leaked more LDH than those with pGGGG, indicated that the leakage was not caused by the production of the recombinant protein, but was more likely due to slow rehabilitation of the plasma membrane after treatment of cells with lipofectamine.

### 3.2. Determination of the length of cultivation before harvesting samples

It was necessary to establish at what time point it was appropriate to harvest the samples, i.e. how long the cells should be allowed to grow after transfection (transiently transfected cells) or induction (stably transfected cell populations). An experiment was therefore performed where samples were harvested at 6, 12, 24, 36 and 48 h after transfection and induction. The results (Fig. 2) showed that *Gussia* luciferase accumulated in the medium and that the production of luciferase seemed to be reduced after 36 h.

In order to be able to compare results between transiently transfected cells and stable populations, it was

found preferable to use sampling procedures as similar as possible. Because luciferase production was high between 24 and 36 h after transfection/induction, and because the wells containing stable populations reached confluency after approximately 30 h, it was decided to harvest samples of transiently transfected cells and stable populations 24 h after transfection and induction, respectively.

### 3.3. *Gussia luciferase* measurements in transiently and stably transfected CHO cells

The first part of the study on the efficiency of luciferase production by CHO cells was done on cells transiently transfected with six plasmid constructs, including pTRE2hyg as a negative control. Cells were transfected, and after changing the medium, the cells were cultivated for 24 h before harvesting samples, and performing activity measurements. The results presented in Fig. 3A, normalised with respect to transfection efficiency, and in Fig. 3B show that transient and

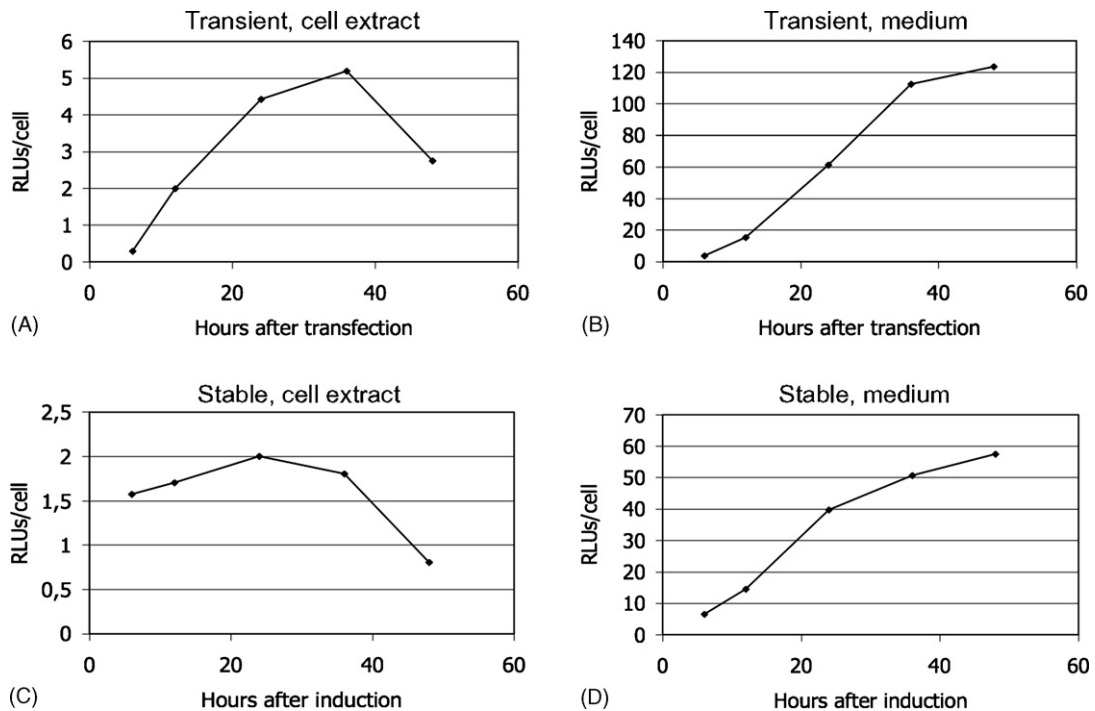


Fig. 2. Activity of *Gussia luciferase* in cells transfected with pGGGG, as a function of time. The graphs represent activity in cell extracts (A) and in medium samples (B) from transiently transfected cells and activity in cell extracts (C) and medium samples (D) from stably transfected cells.

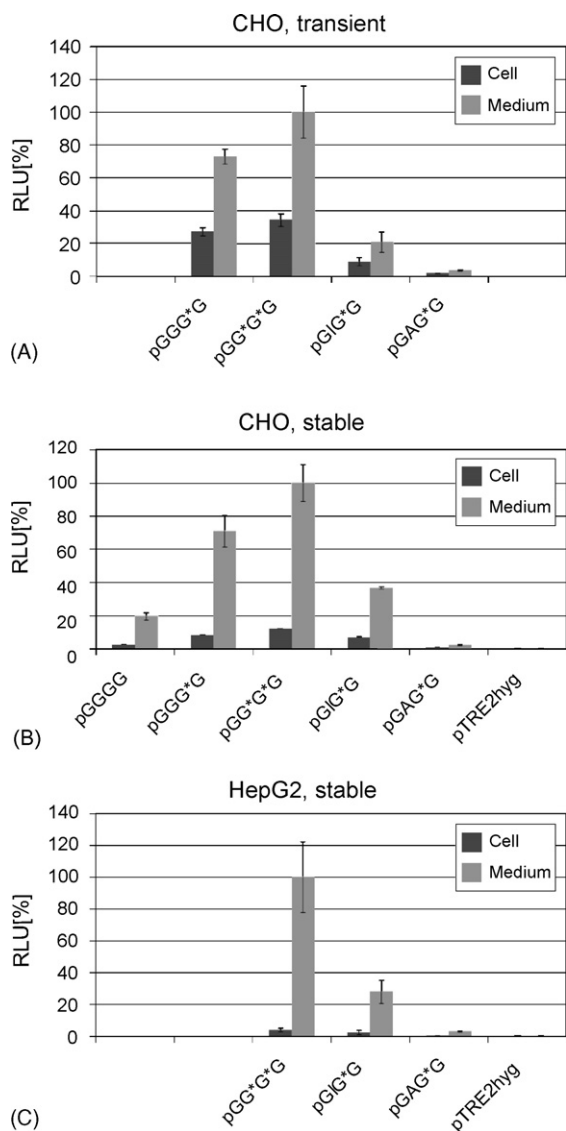


Fig. 3. Luciferase activity profiles in cell extracts and medium samples from transfected CHO and HepG2 cells. (A) Luciferase activity in cell extracts and medium samples from CHO AA8 Tet-Off cells, transiently transfected with the constructs indicated. Values were normalised with those generated from transfection efficiencies obtained by cotransfection with a firefly-luciferase encoding plasmid (pTRE2hygLuc, Clontech Laboratories). Firefly luciferase activity was measured using the Promega “Luciferase Assay System”. The columns represent an average of the results from three independent transfections for each construct with the standard deviations shown. All measurements were calculated in relation to the activity in the medium samples of cells transfected with pGG\*G\*G, which was set to 100%. (B) Luciferase activity measured in cell extracts and medium samples from CHO K1 Tet-On cells stably transfected with

stable transfection give similar patterns of luciferase activity. As seen in Fig. 3B, the levels of luciferase activity were much higher in cells which had been transfected with pGGG\*G and pGG\*G\*G than in cells transfected with pGGGG. The medium samples from cells transfected with pGIG\*G and pGAG\*G displayed luciferase activities of 37 and 2%, respectively, of the activity recorded from cells transfected with the vector containing the *Gaussia* luciferase codon-optimised signal peptide coding region. The low levels of luciferase in the medium were not due to reduced secretion efficiency since the levels in cell extracts were correspondingly low. As expected, no activity was found in cell extracts or medium samples where the cells had been transfected with pTRE2hyg.

It was considered that an explanation for the poor results obtained using the albumin signal peptide could be a lack of cleavage between the albumin signal peptide and the initial amino acids of the *Gaussia* luciferase polypeptide. If the signal peptide was not cleaved off then there was a possibility that this could have severely perturbed enzyme activity. An additional albumin signal-peptide construct was therefore made containing the albumin pro-sequence coding region between the signal peptide and *Gaussia* luciferase coding regions. If the activity of the signal peptidase cleaving off the signal peptide was dependent on upstream and downstream sequences of the cleavage site, it was natural to assume that cleavage would be most efficient when the pro-sequence was present, as this would give albumin sequences on both sides of the cleavage site. There was no danger of a resulting luciferase with the albumin pro-sequence present as an N-terminal fusion peptide, as this polypeptide is known to be cleaved off in the Golgi complex by a process solely depending on a cleavage signal within the pro-sequence (Misumi et al., 1991). The results (data not shown) were identical to the observations when the

the constructs indicated. The columns represent an average of the results from three independent transfections for each construct with the standard deviations shown. All measurements were calculated in relation to the activity in the medium samples of cells transfected with pGG\*G\*G, which was set to 100%. (C) Luciferase activity measured in cell extracts and medium samples from HepG2 cells stably transfected with the constructs indicated. The results are expressed as described in (B) above.

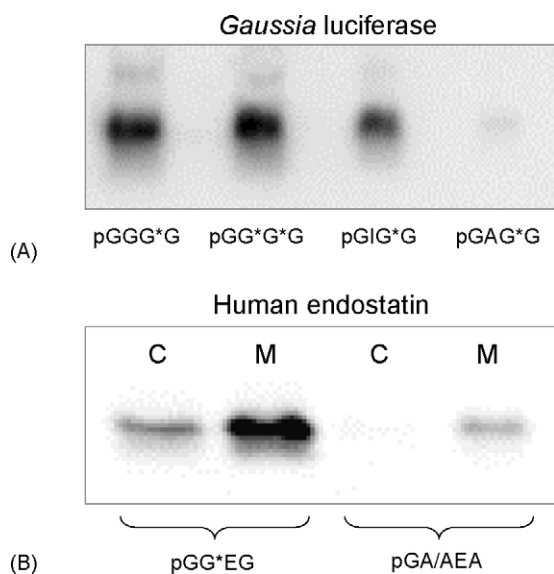


Fig. 4. Immunoblots showing *Gaussia* luciferase and human endostatin production. (A) Immunoblot of medium samples from CHO cells stably transfected with the constructs indicated encoding *Gaussia* luciferase. (B) Immunoblot of cell extracts (C) and medium samples (M) from CHO cells stably transfected with the constructs indicated encoding human endostatin.

pro-sequence was absent and therefore did not indicate that inefficient cleavage afforded an explanation for the low level of luciferase activity recorded using the human albumin signal peptide.

An explanation for the observed low levels of luciferase activity using the Il-2 and albumin signal peptides could have been the presence of a large amount of inactive product. In order to test this possibility Western blotting was performed on medium samples from stably transfected cells. The results presented in Fig. 4A clearly demonstrate that the protein levels mirror the luciferase activity measurements in medium samples, thus ruling out the possibility of the existence of non-active product.

#### 3.4. *Gaussia* luciferase measurements in stably transfected HepG2 cells

As seen in Section 3.3 the human albumin signal peptide was surprisingly poor in terms of *Gaussia* luciferase synthesis and secretion. A possible explanation for the low amount of luciferase produced was that

the albumin signal peptide did not operate satisfactorily in CHO cells, i.e. that it was in the wrong “habitat” and thus functioned sub-optimally. The experiment described in Section 3.3 was therefore repeated using the HepG2 cell line as the host for transfection. The rationale for using this cell line was the fact that HepG2 cells are of hepatic origin and thus should afford a more “true to life” intracellular environment than CHO cells. However, as seen in Fig. 3C, this was not the case. Compared with the *Gaussia* signal peptide that derived from albumin was again far inferior. The same was the case for the Il-2 signal peptide.

#### 3.5. Human endostatin measurements in stably transfected CHO cells

Since the *Gaussia* luciferase signal peptide proved to be far superior to either Il-2 or albumin signal peptides when fused to the coding region of the luciferase protein and tested for luciferase production in transfected CHO cells (Section 3.3), it was important to test whether a protein of mammalian origin could be similarly synthesised in large amounts using the UTRs and the signal peptide of marine origin.

For this purpose human endostatin was chosen as a model protein because of its considerable biomedical potency. This 20 kDa C-terminal proteolytic fragment of the heparan sulphate proteoglycan collagen XVIII, has like several other C-terminal fragments of basement membrane proteins, an anti-angiogenic effect on endothelial cells.

Two additional plasmids were constructed based on pTRE2hyg (see Section 2.1.) with the G 5'UTR and the human endostatin coding region (E) (GenBank accession no. AW080065). One plasmid contained the G\* signal peptide coding region and the G 3'UTR flanking E (pGG\*EG) and the other the human albumin signal peptide and pro-sequence coding region (A/A) and the human albumin 3'UTR (A) flanking E (pGA/AEA). The constructs were stably transfected into CHO cells. Cell extracts and medium samples were tested for human endostatin by immunoblotting. The results are presented in Fig. 4B, and show that the construct containing the signal peptide coding region of *Gaussia* luciferase gave considerably more synthesis of human endostatin than the one containing that derived from albumin.

Table 1  
mRNA and luciferase activity levels do not correlate in CHO cells stably transfected with various signal peptide constructs

Construct	Luciferase mRNA		Luciferase activity (percent related to pGG*G*G)	
	Arbitrary units	Percent related to pGG*G*G	Cell extracts	Medium samples
pGGG*G	313667	117	68	71
pGG*G*G	267634	100	100	100
pGIG*G	237839	89	59	37
pGAG*G	207321	77	5	2

The levels of luciferase mRNA were calculated as arbitrary units based on the Northern blot presented in Fig. 5, using the computer program “Quantity One” (Bio-Rad Laboratories). Values for pGG\*G\*G were set to 100%. The data for luciferase activity were taken from Fig. 3B.

### 3.6. Luciferase mRNA levels in stable CHO cell populations

Based on the observation in Section 3.3 that there were major differences between the performances of individual signal peptides as far as luciferase production in CHO cells was concerned, it was considered that these results could be simply explained by differences between the constructs with respect to the amount of luciferase mRNA present in the cells. In order to test this possibility, total RNA was purified from stably transfected cell populations as described in Section 2.5, and subjected to electrophoresis. Northern blotting and the visualisation of bands were also performed according to the procedures described. The results are presented in Fig. 5. It can be seen that there were substantial amounts of *Gaussia* luciferase mRNA in cells transfected with pGGG\*G, pGG\*G\*G, pGIG\*G and pGAG\*G. As expected, transfection with pGGGG

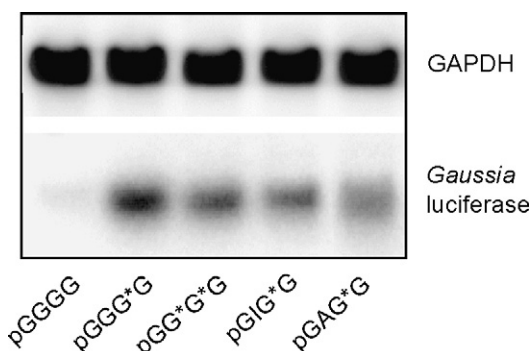


Fig. 5. Northern blot showing the levels of GAPDH mRNA and codon-optimised *Gaussia* luciferase mRNA in total RNA samples. The RNA was isolated from stable populations of CHO cells, transfected with the constructs indicated. The membrane was first probed for *Gaussia* luciferase mRNA, and then following stripping, for GAPDH mRNA.

(native codon usage) only resulted in a very weak band, proving that the probe hybridised with strict specificity.

A reference probe was used to monitor the level of the mRNA of a housekeeping gene, namely that of GAPDH, an essential glycolytic pathway enzyme (Thellin et al., 1999). The results showed that the levels of GAPDH mRNA were approximately the same in all cell populations.

The levels of luciferase mRNA were quantified and this data together with luciferase activity data obtained from stably transfected cell populations (taken from Fig. 3B) are presented in Table 1. In contrast to the large differences observed in the luciferase content in cell extracts and medium samples, the levels of mRNA in the stably transfected cell populations did not vary greatly. It can be seen from Table 1 that transfection with pGAG\*G gave a level of luciferase mRNA which amounted to 77% of that observed with pGG\*G\*G, but only 5% of the luciferase activity was recorded in cell extracts and 2% in medium samples. It was thus apparent that although transfection with pGAG\*G gave rise to a substantial quantity of luciferase mRNA, only a very limited amount of active enzyme was produced. Transfection with pGIG\*G gave intermediary values. The results were surprising in that the only difference between the individual constructs was the nature of the coding sequence of the signal peptide.

## 4. Discussion

The major objective of the work was to replace the native signal peptide coding region in the mRNA encoding the reporter protein *Gaussia* luciferase, with the coding regions of two other signal peptides in order to study whether or not luciferase synthesis and secretion was modulated to any great extent by the different sequences employed. Signal peptides derived

from human Il-2 and albumin were chosen as alternatives to the *Gaussia* luciferase signal peptide because both these proteins are known to be secreted with great efficiency from mammalian cells. Interleukin-derived signal peptides are in fact widely used in both commercial protein production and in gene therapy research (Bamford et al., 1998; Komada et al., 1999; La Flamme et al., 1995; Liu et al., 1997; Sasada et al., 1987, 1988; Suzuki et al., 2001), presumably because of their reputation as being very efficient. A seamless cloning procedure was used during generation of constructs to avoid the inclusion of any linker sequences within the mRNA.

The luciferase activity assay showed that both transiently and stably transfected CHO cells displayed a higher amount of luciferase in the medium where the codon usage of the *Gaussia* luciferase signal peptide had been optimised. As expected, optimisation of the luciferase coding region led to a major increase in luciferase production. These results underline the importance of favourable codon usage when a recombinant protein is to be produced in a foreign host cell.

Lactate dehydrogenase measurements in the medium collected from transiently transfected cells showed substantial activity above background levels caused by the presence of LDH in FBS. This was a clear indication that leakage of cytosolic content had indeed occurred. It is thus evident that the plasma membrane of lipofectamine treated cells requires time to recover from perturbation caused by the agent. It is important to bear this in mind when studying secretion efficiency at early times in a transiently transfected cell population since mere leakage may be interpreted incorrectly as secreted product. A certain level of caution is therefore required when assessing results from transient transfection if the issue of leakage has not been adequately taken into account.

Surprisingly, the use of signal peptides from Il-2 and albumin gave rise to much lower levels of synthesis and secretion of the recombinant protein compared to when the corresponding *Gaussia* luciferase sequence was used. It was unexpected that the signal peptide of marine origin should function far better in a mammalian cell than the two sequences derived from human proteins. Furthermore, the fact that the human albumin signal peptide was extremely ineffective was difficult to explain in the light of its important role in hepatic

cells. Substantial amounts of albumin are synthesised and secreted from the liver into the blood circulation every day. In clear contrast to the “normal” physiological conditions under which the signal peptides of human origin operate, the copepod lives at 4 °C and under high pressure in deep sea water. The signal peptide derived from *Gaussia* luciferase thus functions in a completely different environment.

Since the human albumin signal peptide performs poorly in CHO cells it was considered likely that the sequence may well function more effectively in a cell line of liver origin. An experiment was therefore carried out where a *Gaussia* luciferase signal-peptide construct and an albumin signal-peptide construct were transfected into HepG2 cells and both cell extracts and medium samples were assayed for *Gaussia* luciferase activity. The results were virtually identical to those obtained using CHO cells, and were interesting in two ways, firstly in that the albumin signal peptide did not operate more efficiently in a liver cell line than in CHO cells, and secondly, that a signal peptide derived from a marine organism was extremely effective not only in a hamster but also in a human cell line.

Interestingly, Zhang et al. (2005) have recently shown that the secretion of both alkaline phosphatase and endostatin could be increased by up to 3.5-fold in mammalian cells by increasing the basicity and the hydrophobicity of the Il-2 signal peptide. It is therefore evident that the performance of the Il-2 signal peptide can be improved substantially.

The fact that the luciferase signal peptide derived from *Gaussia*, a marine copepod, was far superior to two mammalian signal peptides in CHO and HepG2 cells with respect to recombinant protein production was quite unexpected. Notable was the observation that replacement of the signal peptide coding region from *Gaussia* with that either from Il-2 or albumin did not have a major effect on the amount of mRNA transcript since the corresponding levels were 89 and 77%, respectively. It was thus evident that the dramatic impact on the quantity of active recombinant protein was not merely due to a lack of mRNA but rather connected to a post-transcriptional event. Why there was a substantial quantity of mRNA transcript in the transfected cells but it was apparently poorly utilised is not yet known. The observations suggest that more important than quantity is the actual nature of the

quality of the transcript and the cell's ability to utilise it in an efficient manner.

Since neither II-2 nor albumin signal peptides matched the performance of the native *Gaussia* signal peptide with respect to *Gaussia* luciferase synthesis/secretion, it was important to test the ability of this peptide to function in the synthesis of another reporter protein. For this purpose the luciferase of *Vargula hilgendorfi*, an ostracod crustacean, was chosen. The *Gaussia* signal peptide proved to be far more effective than the native *Vargula* luciferase signal peptide in producing active *Vargula* luciferase (manuscript in preparation). It was thus evident that the native signal peptide is not necessarily the most effective. That a signal peptide of non-mammalian origin could be used effectively in the production of a mammalian protein was demonstrated in the experiment where it was shown that human endostatin synthesis was augmented when the albumin signal peptide was replaced with that derived from *Gaussia* luciferase. Taken together the results suggest that the *Gaussia* luciferase signal peptide has extremely interesting properties (patent application no. PCT/GB2004/002779 "Protein Expression System").

The results presented here show quite clearly that the choice of signal peptide is crucial when constructing a "secretion cassette" with the aim of optimising synthesis/secretion of a recombinant protein. To our knowledge this is the first report where comparison has been made between a signal peptide of marine origin and two widely used signal peptides derived from mammalian sources. Future work will be designed to test other signal peptides of marine origin in order to investigate whether or not it is possible to boost recombinant protein production even further.

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