UTRtech™: Exploiting mRNA Targeting To Increase Protein Secretion From Mammalian Cells

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Abstract: The technology of UniTargetingResearch AS (UTRtech™) is based on the finding that the efficiency of directing mRNA to the endoplasmic reticulum is influenced by targeting signals. Using selected signals, genetically engineered mammalian cells are generated from which a protein of interest can be efficiently secreted. An industrial collaboration has revealed that UTRtech™ has the potential to significantly enhance the production of therapeutic proteins.

Keywords: CHO cells, IgG supersecretion, recombinant protein, seamless cloning, signal peptide, 3'UTR.

1. INTRODUCTION

In order to make mammalian cell systems competitive as cell factories it is of extreme importance to overcome the problem of low levels of protein production. Numerous approaches have been applied during recent years where attention has been paid mainly to modifying the cell's growth conditions (media composition and process control) and increasing the transcriptional activity of the recombinant gene (e.g. utilisation of strong promoters/enhancers in the expression vector, amplification of gene copy number) (Wurm, 2004). A new approach is currently being developed in our laboratory where the focus is on aspects of post-transcriptional events. We

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have earlier shown that polysomes in CHO cells can be fractionated into free, cytoskeletal-bound and membrane-bound populations (Pryme *et al.*, 1996). Based on our finding that the efficiency of directing mRNA to membrane-bound polysomes and thus to the endoplasmic reticulum, is dependent on the presence of targeting signals (specific signal peptides and 3'UTRs) (Partridge *et al.*, 1999), we have addressed our efforts to the improvement of protein synthesis/secretion by exploiting these results. We have compared the relative efficiencies of different signal peptides and signal peptide/3'UTR "doublets" from various sources on the production of a model protein (a marine luciferase). This led to the development of UTRtech™. In collaborative experiments with an industrial partner we have tested the technology for its ability to promote increased immunoglobulin (IgG) production. Using the same approach we have investigated the possibility to secrete an intracellular model protein (EGFP). The results from these studies are reported here.

2. Materials And Methods

2.1 Cell culture and transfection

CHO cells were grown in monolayer culture using DMEM medium containing 10% FBS where appropriate, and 100 U/ml penicillin. Cells were incubated in a humified atmosphere of 5% CO_2 at 37^{o}C. Transfection was performed using lipofectamine and stable cell populations were established within 4 weeks using a selection medium.

2.2 Seamless cloning

All vector constructs were made using a seamless cloning strategy. This PCR-based method was developed in our laboratory for the directional insertion/substitution of DNA, independent of restriction enzyme sites and avoiding the incorporation of linker sequences.

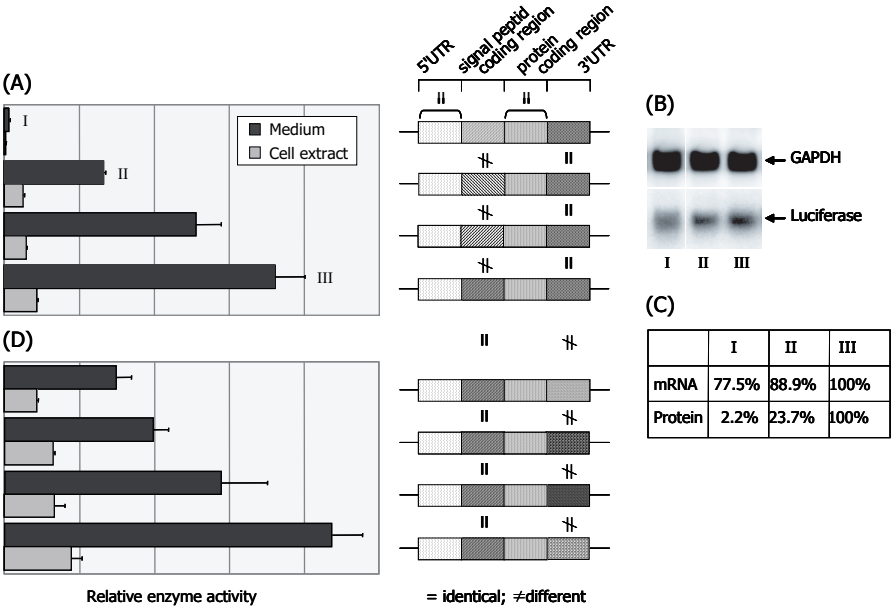
2.3 Bioluminescence assay, ELISA, Western and Northern blotting

These were carried out according to routine laboratory procedures.

3. Results

3.1 Defining Utrtech™ Using A Model Protein

Using a naturally secreted luciferase from the marine copepod *Gaussia princeps* as a model protein, a number of signal peptides have been tested for their ability to promote synthesis and secretion of this protein in transfected CHO cells. Figure 1A shows the relative efficiencies of selected signal peptides with respect to the amounts of luciferase produced and recovered in the growth medium. The amounts differ by factors of up to 50.



	I	II	III
mRNA	77.5%	88.9%	100%
Protein	2.2%	23.7%	100%

Figure 1. A) Effect of different signal peptides on synthesis/secretion of Gaussia luciferase. Luciferase activity in culture medium and cell extracts of stably transfected CHO cell populations was measured with a standard bioluminescence assay and corrected according to cell number. The signal peptides used are derived from the following proteins (top to bottom): human albumin, human interleukin-2, human trypsinogen, Gaussia luciferase. B) Northern blot analysis of mRNA isolated from selected CHO cell populations in (A), designated I-III. DNA probes against GAPDH (control) and luciferase coding region were used. C) Quantification of luciferase mRNA bands in (B) using QuantityOne software (Bio-Rad). Both mRNA measurements and luciferase activity measurements (medium values from panel A) are related to the maximal values achieved using the Gaussia-luciferase signal peptide (set as 100%). D) Effect of various 3'UTRs combined with the Gaussia-luciferase signal peptide on synthesis/secretion of Gaussia luciferase.

In order to eliminate the possibility that the striking differences observed were due to varying levels of mRNA, Northern blot analysis was performed (Figure 1B). The levels of mRNA and secreted luciferase were quantified (Figure 1C). Interestingly, although the amounts of luciferase mRNA where the albumin signal peptide was used, is about 80% of that found with the *Gaussia*-luciferase signal peptide, the level of secreted luciferase is only 2%.

In a further set of experiments the *Gaussia*-luciferase signal peptide was tested in combination with various 3'UTR sequences derived from genes coding for secreted proteins. As exemplified in Figure 1D, again a significant variation in the levels of luciferase secretion was observed. Efficient signal peptide/3'UTR doublets were identified. The doublets together with the seamless cloning technology to be used for the construction of "secretion cassettes" containing the coding region of a protein of interest is defined as UTRtech™.

3.2 Proof of Principle: Supersecretion of Igg

In a collaborative project with Angel Biotechnology Ltd aiming to boost human IgG production, we have compared the effectiveness of an optimised signal peptide/3'UTR doublet with those doublets originally present in the vector system determined by the company. As shown in Figure 2 there is a clear increase in intact IgG production when using UTRtech™, both in a stably transfected CHO cell population (Panel A) and when testing about 200 randomly selected single CHO cell clones (Panel B). The mean titre of the clones is more than 50% higher when transfected with the modified *versus* unmodified vector. Clones producing maximal amounts of IgG were observed in the range of 200-250 mg/ml using UTRtech™ compared to clones producing 80-90 mg/ml with the original vector not containing the optimised signal peptide/3'UTR doublet.

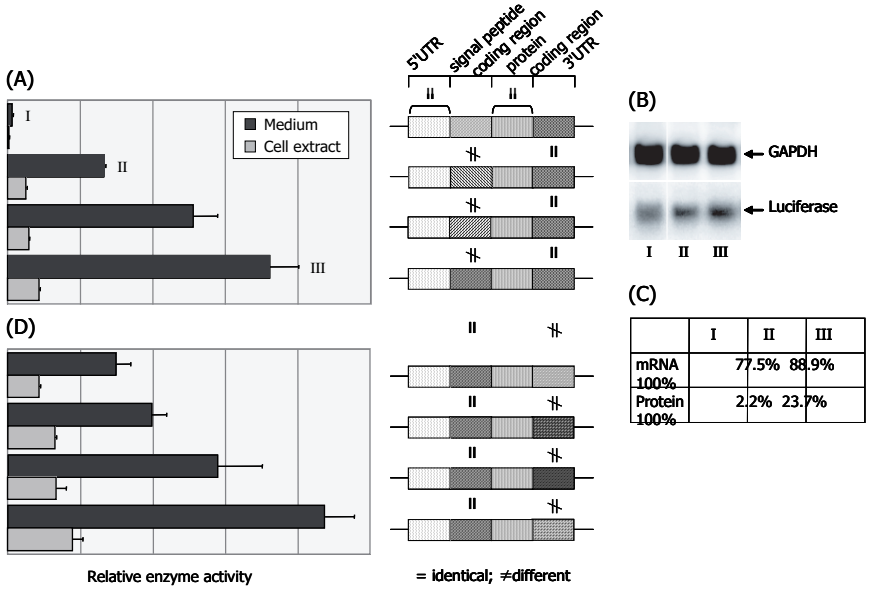


Figure 2. A) Assembled IgG identified in the medium of stably transfected CHO cell populations. B) Assembled IgG identified in the medium of randomly selected single clones from transfected CHO cells (work performed by Angel Biotechnology Ltd). In (A) two vectors encoding either the IgG heavy or light chain were cotransfected, whereas in (B) both chains were encoded by the same vector. In (A) medium samples were adjusted according to total protein concentration and subjected to Western blot analysis. In (B) medium samples were subjected to ELISA. "Partner" indicates the original and "UTRtech" the modified vector(s).

3.3 Secretion of an Intracellular Protein

UTRtech™ has been employed to successfully secrete an intracellular model protein, namely EGFP (Figure 3). Further, the experiment demonstrates the importance of the seamless cloning technology in order to produce authentic protein. When comparing the result obtained with our secretion vector and the result obtained with a commercially available secretion vector where cloning requires the use of restriction enzymes, it is evident that the presence of linker sequences is disadvantageous.

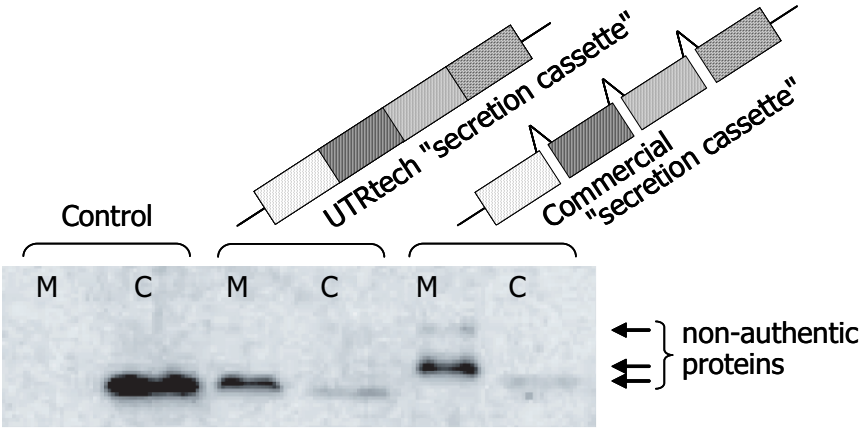


Figure 3. Western blot analysis of EGFP in the culture medium (M) and cell extracts (C) of CHO cell populations stably transfected with vector constructs containing the "cassettes" specified. The different genetic components are designated in Figure 1. The “hooks” in the commercial secretion vector indicate linker sequences.

4. Discussion

The observations we made when testing individual signal peptides were very surprising in that we had expected that the signal peptide of albumin, a protein produced and secreted constitutively in large quantities by the liver, would be extremely effective with regards to production of recombinant protein in mammalian cells. The amount of luciferase recovered in the culture medium when using the albumin signal peptide, however, was only 2% of the amount achieved using the signal peptide from *Gaussia* luciferase. Interestingly, similar results as with CHO cells were obtained with HepG2 cells. Also other signal peptides of human origin expected to perform well (e.g. interleukin-2, trypsinogen) did not reach the same high level of recombinant protein secretion achieved using the marine signal peptide. Since the levels of mRNA measured in the samples from CHO cells transfected with the albumin/interleukin/*Gaussia*-luciferase signal peptide constructs vary to a much lesser extent than the corresponding levels of the secreted luciferase, the results would strongly suggest that the effect is at the post-transcriptional level. This is supported by preliminary results obtained from a collaboration with Selexis, a company focusing on transcriptional enhancement by employing specific DNA elements binding to nuclear scaffolding. Combining UTRtech^{TM} with their technology (MARtech™) resulted in a 100% increase in luciferase secretion, demonstrating that

UTRtech™ is complementary to approaches aiming at improved recombinant protein production through elevated mRNA levels.

Since the results had been obtained using a model reporter protein, it was important to ascertain whether or not similar observations could be made with a protein of commercial interest. Collaborative experiments performed together with Angel Biotechnology Ltd indeed showed that incorporating UTRtech™ into their expression system significantly enhanced IgG production on the laboratory scale.

Further collaborative experiments performed with academic/industrial partners have demonstrated that in addition to recombinant protein production, UTRtech™ has the potential to be adaptable for use in other applications such as gene and cell therapy where efficient protein secretion is imperative.

Regarding the successful application of UTRtech™ to secrete an authentic intracellular protein, this has important implications e.g. for the production of certain "difficult-to-express" proteins which exhibit toxicity when accumulating in the host cell. Their secretion would circumvent the problem of cell damage/death.

The results described here clearly illustrate the fact that mammalian signal peptides hitherto thought to be extremely effective with regard to recombinant protein production (e.g. the signal peptide from albumin), can be replaced by far more efficient sequences originating from non-mammalian sources. Further, they demonstrate the importance that the nature of the 3'UTR is also taken into consideration such that the most effective signal peptide/3'UTR doublet is chosen for the recombinant protein whose production rate is to be boosted.

5. Summary

1. The choice of signal peptide/3'UTR doublet is imperative when one has the goal of achieving improved yields of recombinant protein in mammalian cells.
2. The adaptation of seamless cloning technology results in the production of authentic protein.
3. UTRtech™ can be used to enhance the production of naturally secreted proteins and to secrete intracellular proteins.
4. Industrial collaborations have demonstrated the incremental nature of UTRtech™.
5. UniTargetingResearch AS (www.unitargeting.com) is the sole supplier and IP holder of UTRtech™.

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