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Quantitative analysis of transcription and translation in gene amplified Chinese hamster ovary cells on the basis of a kinetic model

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Abstract

The elevation of expression levels for secreted glycoproteins by gene amplification in mammalian cells shows a saturation behavior at high levels of gene amplification. At high expression levels a drop in the secretion efficiency for the recombinant protein occurs (Schröder and Friedl, 1997), coinciding with the appearance of misfolded protein in the cell. In this communication we investigated whether additional limitations exist at the levels of transcription and translation. Four Chinese hamster ovary (CHO) cell lines expressing different amounts of human antithrombin III (ATIII) were used as a model system. A tenfold increase in the ATIII cDNA copy number from the lowest to the highest producing cell line coincided with a 38-fold increase in ATIII mRNA levels, and an 80-fold increase in the amount of intracellular ATIII levels. The data was analyzed using a simple kinetic model. The following conclusions were derived: I. The transcriptional activity for the recombinant protein is not saturated. II. Translation itself is not saturated either, but may be downregulated as secretion efficiency drops. III. Two explanations for the previously reported drop in secretion efficiency for the recombinant protein with increasing expression level are possible: A. Protein degradation is an alternative fate for translated ATIII and the fraction of ATIII degraded after translation increases as expression level is increased. B. Translation is downregulated as the secretory apparatus becomes exhausted to maintain cell viability.

Abbreviations: APC, activated protein C; ATIII, antithrombin III; BiP, heavy-chain binding protein; bp, base pair; cDNA, complementary 2′-deoxyribonucleic acid; CHO, Chinese hamster ovary; DHFR, dihydrofolate reductase; DIG-[11]-dUTP, digoxigenin-*O*-succinyl-ϵ-aminocaproyl[5-(3-aminoallyl)-2′-deoxyuridine-5′-triphosphate; ELISA, enzyme-linked immunosorbent assay; ER, endoplasmic reticulum; GRP, glucose-regulated protein; GS, glutamine synthetase; mRNA, messenger ribonucleic acid; MSX, methionine sulfoximine; MTX, methotrexate; PKR, double-stranded RNA-activated eukaryotic initiation factor (eIF)-2α kinase

Symbols: c_{cDNA}, intracellular cDNA concentration; c_{mRNA}, intracellular mRNA concentration; $c_{protein,inside}$, intracellular protein concentration; $c_{protein,outside}$, protein concentration in the culture medium; k_1, transcriptional rate coefficient; k_2, translational rate coefficient; k_3, secretory rate coefficient; k_{-1}, rate coefficient for mRNA degradation; k_{-2}, rate coefficient for intracellular protein degradation; η, transcription efficiency; μ, specific growth rate; ξ, translation efficiency; ζ, secretion efficiency

Introduction

The amplification of genes by stepwise increasing the resistance of mammalian cells to certain toxic chemicals as methotrexate (MTX) or methionine sul-

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foximine (MSX) is a powerful technique for the engineering of mammalian cells overexpressing valuable therapeutic proteins as erythropoietin, factor VIII, and tissue plasminogen-activator (Kane, 1991; Kaufman, 1990, 1993; Kellems, 1991). At high levels of gene amplication no further increase in the expression level of α-amidating enzyme in CHO cells has been observed (Miller et al., 1992) and the increase in ATIII production by CHO cells decreased as the expression level increased (Zettlmeissl et al., 1987). Guarna et al. (1995) have shown that an optimal cDNA gene dosage exists for the transient production of activated protein C (APC) in CHO cells. This data is very similar to data reported on the influence of plasmid content in *Escherichia coli* (Aiba et al., 1982; Bailey et al., 1983; Seo and Bailey, 1985, 1986; Yamakawa et al., 1989) on protein production and shows that the productivity achievable by gene amplified mammalian cells is limited by steps involved in gene expression.

In a previous work we have demonstrated that the secretion efficiency for ATIII in CHO cells decreases steadily as the expression level increases. Only at high expression levels inactive ATIII was detected in the cells, indicating that maturation of ATIII to its active form becomes limiting at high expression levels (Schröder and Friedl, 1997). Here we investigated whether transcription of ATIII cDNA genes or translation of ATIII mRNA becomes inefficient at elevated expression levels. Two different scenarios are imaginable: I. Both steps of gene expression become rate-limiting *per se* as the ATIII cDNA gene dosage or ATIII mRNA level are increased by gene amplification beyond a threshold concentration that the cell cannot handle efficiently. II. The decreased secretion efficiency or the presence of misfolded ATIII in the endoplasmic reticulum (ER) may downregulate transcription or translation. It has been shown that the accumulation of unfolded proteins within the ER induces a stress response in mammalian cells which involves the induction of genes for the heavy-chain binding protein (BiP), the glucose-regulated protein of 94 kDa, protein disulfide isomerase, and calnexin (Rowling and Freedman, 1993). Under these conditions an inhibition of initiation of translation occurs (Brostrom et al., 1996; Srivastava et al., 1995) by activating the double-stranded RNA-activated eukaryotic initiation factor (eIF)-2α kinase (PKR) (Prostko et al., 1995). As highly gene amplified cells are still viable, a mechanism must exist which prevents the accumulation of immature recombinant protein to infinite levels in the cell, a condition which would require infinite amounts of ATP and therefore contradicts cell viability. One could be downregulation of either transcription or translation. Another possibility is that protein degradation within or near the ER becomes a major fate of the translated recombinant protein as has been reported for hepatitis B surface antigen (Pendse et al., 1992).

On the basis of a modification of a kinetic model first presented for gene expression in *E. coli* by Ryu et al. (1991) we investigated whether transcription and translation efficiencies are decreased at high expression levels or if protein degradation is an alternative fate for translated but not secreted recombinant protein within the secretory pathway.

Materials and methods

Materials

The suppliers for materials used in cell culture and for protein chemistry have been listed elsewhere (Schröder and Friedl, 1997). In addition, the following materials were used for molecular biology methods: Plasmid pSVATIII (Zettlmeissl et al., 1987) was a kind gift of Dr. G. Zettlmeissl (Chiron Behring, Marburg, Germany), and the strain *Escherichia coli* DH5α a generous gift of Dr. H. G. Gassen (Technical University of Darmstadt, Darmstadt, Germany). Agarose was purchased from Sigma (Deisenhofen, Germany), the DIG DNA labeling and detection kit and Lumigen PPD® from Boehringer Mannheim (Mannheim, Germany), positively charged Hybond-N+ nylon membranes from Amersham Buchler (Braunschweig, Germany), the QIAGEN® plasmid kit from QIAGEN (Hilden, Germany), restriction endonucleases *Hind*III, *Sal*I, and *Xba*I from Life Technologies (Eggenstein, Germany), and the SpinBind® DNA recovery system for agarose gels from Biozym Diagnostik (Hessisch Oldendorf, Germany). All other chemicals were of analytical grade and were purchased either from Fluka (Neu-Ulm, Germany), Roth (Karlsruhe, Germany) or Sigma (Deisenhofen, Germany).

Cell culture and determination of ATIII

The DHFR negative CHO cell line DUKXB1 (Urlaub and Chasin, 1980) and the ATIII secreting cell lines CHO-A11-A2, CHO-A11-A27, CHO-A11-A279, and CHO-A11-A279-C7 (Schröder and Friedl, 1997) were a kind gift of Dr. G. Zettlmeissl (Chiron Behring, Marburg, Germany). The ATIII-secreting cell lines were

derived from cell line CHO DUKXB1 by cotransfection with plasmids pSV0ADHFR and pSVATIII (Zettlmeissl et al., 1987) and subsequent selection for growth in a medium lacking glycine, thymidine, and hypoxanthine. Selection for increasing resistance to MTX yielded the cell lines CHO-A11-A2 (resistant to 0.1 μM MTX), CHO-A11-A27 (resistant to 1.0 μM MTX), CHO-A11-A279 (resistant to 10 μM MTX), and CHO-A11-A279-C7 (resistant to 100 μM MTX). The cultivation of the cells has been described previously (Schröder and Friedl, 1997).

Molecular biology methods

Competent *E. coli* DH5α were transformed with plasmid pSVATIII by a modification of the method of Hanahan (1983). Plasmid pSVATIII was isolated, digested with restriction endonucleases *Xba*I and *Sal*I to yield a 1509 base pair (bp) large fragment containing the ATIII cDNA, the fragment eluted from agarose gels and labeled by random priming with digoxigenin-*O*-succinyl-ϵ-aminocaproyl[5-(3-amino-allyl)-2′-deoxyuridine-5′-triphosphate (DIG-[11]-dUTP) as recommended by the manufacturer. Genomic DNA (Sharma et al., 1993) and total RNA (Chomcyznski and Sacchi, 1987) were isolated from a confluent 75 cm^2 T-flask of CHO cells. Genomic DNA was simultaneously digested with 1.25 units per μg DNA *Hind*III and *Xba*I to liberate the ATIII cDNA. The digestion reaction was incubated for 3 h at 37 °C, the same amount of restriction endonucleases added and incubated for further 3 h at 37 °C. Size separation of total RNA was done in formaldehyde agarose gels (Tsang et al., 1993), blots were hybridized according to Engler-Blum et al. (1993) with 4 ng mL^{-1} of the DIG-[11]-dUTP labeled 1509 bp *Xba*I/*Sal*I fragment of pSVATIII and the hybridized probe detected using the chemiluminescent substrate Lumigen PPD® for alkaline phosphatase (Engler-Blum et al., 1993). All other molecular biology methods were essentially performed as described previously (Sambrook et al., 1989). Densitometry was done using a Flatbed Color IIHR scanner (Vobis, Darmstadt, Germany) and the computer program Micrografx® Picture Publisher 6.0 (Micrografx, Munich, Germany). Signals on scanned blots were quantitated using the program QuantiScan from Biosoft (Cambridge, U.K.).

Results

General considerations

The levels of messenger RNAs (mRNA) and intracellular proteins are tightly regulated by processes involving the synthesis and degradation of each species. On this basis a kinetic model has been developed for the production of proteins in *E. coli* (Ryu et al., 1991). This model is easily adapted to the production of secretory proteins by mammalian cells if secretion of the protein is considered (Figure 1). Protein degradation in the cell has been previously considered to be a first-order process (Noe and Delenick, 1989), but may also be initiated by aggregation of the protein in the ER (Kiefhaber et al., 1991) which has been shown to be approximately a second order process for lactate dehydrogenase *in vitro* (Zettlmeissl et al., 1979). From this model the following rate equations for the net change in the level of a particular mRNA (1), the level of a particular intracellular protein (2a) or (2b), and the secretion rate (3) are obtained:

$$\frac{dc_{mRNA}}{dt} = k_1 \eta c_{cDNA} - k_{-1} c_{mRNA} - \mu c_{mRNA} \quad (1)$$

$$\frac{dc_{protein,inside}}{dt} = k_2 \xi c_{mRNA} - k_{-2} c_{protein,inside} - k_{-3} \zeta c_{protein,inside} - \mu c_{protein,inside} \quad (2a)$$

$$\frac{dc_{protein,inside}}{dt} = k_2 \xi c_{mRNA} - k_{-2} c^2_{protein,inside} - k_{-3} \zeta c_{protein,inside} - \mu c_{protein,inside} \quad (2b)$$

$$\frac{dc_{protein,outside}}{dt} = k_3 \zeta c_{protein,inside} \quad (3)$$

Transcription (η), translation (ξ), and secretion (ζ) efficiencies have been included to describe possible non-linear effects. For a viable cell with stable expression of a recombinant protein steady state conditions have to exist for the intracellular concentrations of the corresponding mRNA and the intracellular protein. In other cases, either expression would cease, which is contrary to the requirement that the expression of the transferred gene is stable, or the intracellular species would accumulate to infinite levels, a condition contradicting the viability of the cell. Thus, the following relationships are derived:

$$c_{mRNA} = \frac{k_1 \eta}{k_{-1} + \mu} c_{cDNA} \quad (4)$$

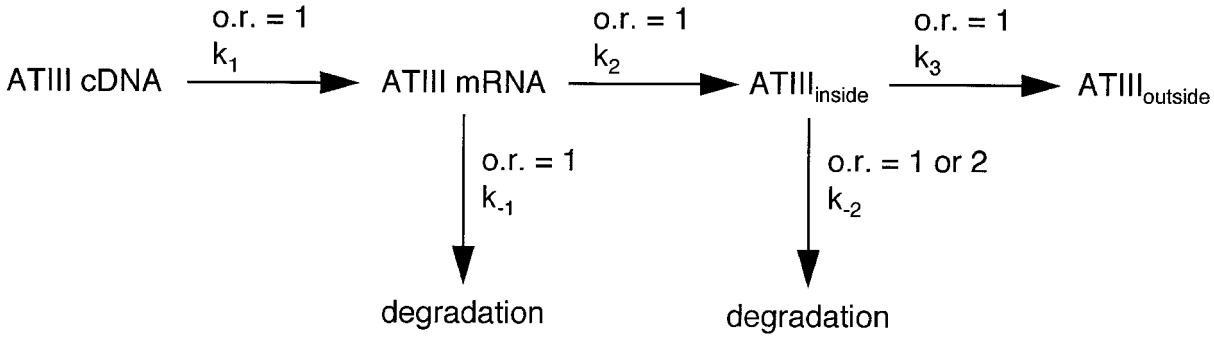

Figure 1. Kinetic model for recombinant protein secretion. ATIII is depicted as an example for a secreted recombinant protein. k_1, k_2, and k_3 are the rate coeffcients for the synthesis of ATIII mRNA, the intracellular ATIII protein, and the secreted ATIII, respectively. k_{-1} and k_{-2} are rate coefficients for the intracellular degradation of the ATIII mRNA and the intracellular ATIII protein, respectively. The order of reaction (o.r.) for each step of gene expression is shown above the corresponding rate coefficient and was taken from Ryu et al. (1991). Degradation of the intracellular ATIII may be a first or second order process (see 'Results').

$$c_{protein,inside} = \frac{k_2\xi}{k_{-2} + \mu} c_{mRNA} \quad \text{or}$$

$$c_{protein,inside} = \left(\frac{k_2\xi}{k_{-2}} c_{mRNA}\right)^{0.5} \qquad (5)$$

$$c_{protein,inside} = \frac{k_2\xi}{k_3\zeta + \mu} c_{mRNA} \qquad (6)$$

Equations (5) apply if secretion is negligible compared to intracellular aggregation and degradation of the protein. The left equation is derived for first-order protein degradation and the right for second-order protein degradation. Equation (6) results if protein degradation is negligible compared to secretion. From these equations it can be concluded that the amount of mRNA is proportional to the amount of cDNA copies in the genome, and the amount of intracellular protein is proportional to the amount of mRNA copies or to its square root. Deviations from linearity should indicate a change in transcription and translation efficiencies, which in turn is then related to expression level. On the basis of these conclusions we were interested whether transcription or translation efficiencies are influenced by expression level. Therefore we determined the ATIII cDNA copy number, and the ATIII mRNA level of recombinant CHO cells expressing different amounts of ATIII. The amount of intracellular ATIII for these four cell lines has been reported previously (Schröder and Friedl, 1997). The quantitative relationship between each molecular precursor and successor for each level of gene expression was then compared. To exclude influences from different specific growth rates all experiments were done with stationary cultures of CHO cells. The parental cell line CHO DUKXB1 of these cells was included in all experiments as a negative control.

Analysis of the transcriptional level

The transcriptional level was analyzed by comparing the relative amount of ATIII cDNA copies per cell with the relative amount of ATIII mRNA per cell.

Determination of relative ATIII cDNA copy number per cell

For each cell line 8 μg genomic DNA were digested with *Hind*III and *Xba*I as described under 'Materials and Methods' to obtain a fragment that consisted only of the ATIII cDNA. The digested genomic DNA was loaded onto an agarose gel, separated by size, stained with ethidium bromide (Figure 2A), blotted onto a Hybond-N+ nylon membrane by capillary transfer, and hybridized with the DIG-[11]-dUTP labeled ATIII cDNA prepared from plasmid pSVATIII as described under 'Materials and Methods' (Figure 2B). An ATIII cDNA standard prepared from plasmid pSVATIII was included to verify that all signals were within the linear range of the signal intensity. The regression coefficient of this standard curve was 0.987. The signals were scanned and the signal intensity related to the number of cells loaded unto each lane of the gel. The amount of cells loaded onto each lane was calculated as follows: First, the actual amount of digested genomic DNA loaded onto each lane had to be corrected, because Figure 2A showed different intensities in ethidium bromide fluorescence for each lane. This variation is due to pipetting errors which are caused by the high viscosity of genomic DNA solutions. We

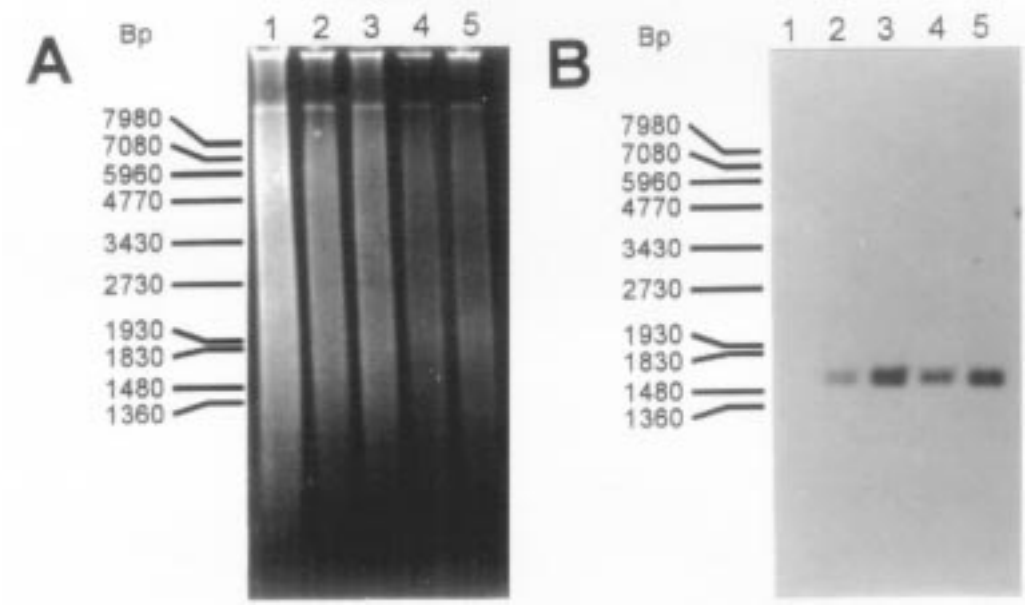

Figure 2. Southern blot analysis of the ATIII cDNA copy number in gene amplified CHO cells. (A) Agarose gel stained with ethidium bromide before capillary transfer took place. (B) Southern blot analysis of this gel with DIG-[11]-dUTP labeled ATIII cDNA prepared from plasmid pSVATIII as described under 'Materials and Methods'. 8 μg of genomic DNA from CHO DUKXB1 (lane 1), CHO-A11-A2 (lane 2), CHO-A11-A27 (lane 3), CHO-A11-A279 (lane 4), and CHO-A11-A279-C7 cells (lane 5) were digested with *Hind*III and *Xba*I and loaded onto the gel.

therefore calculated the relative amount of genomic DNA loaded onto each lane on the basis of the lane which showed the highest fluorescence in the ethidium bromide stain. Second, from these corrected values the amount of cells loaded onto each lane was calculated using the yield of the genomic DNA preparations. Per 10^6 cells the amount of genomic DNA prepared from each cell line varied considerably: 25 μg for CHO-A11-A2, 27 μg for CHO-A11-A27, 107 μg for CHO-A11-A279, and 71 μg for CHO-A11-A279-C7 cells. This probably reflects variable yields of the DNA isolation procedure. To calculate the relative amount of ATIII cDNA copies the ATIII cDNA copy number of CHO-A11-A2 cells was arbitrarily set to a value of 1.0 and the relative amount of ATIII cDNA copies of the other three cell lines related to CHO-A11-A2 cells.

Determination of relative ATIII mRNA copy numbers per cell

The relative amount of ATIII mRNA per cell in the four cell lines was determined by densitometry from the Northern blot shown in Figure 3B essentially as described above. The yields of the total RNA preparations per 10^6 cells were 10 μg for CHO-A11-A2, 22 μg for CHO-A11-A27, 25 μg for CHO-A11-A279, and 42 μg for CHO-A11-A279-C7 cells. The actual amount of loaded RNA was corrected by scanning and quantifying the signal intensity of the ethidium bromide stained bands of the 28 S ribosomal RNA (Figure 3A). Plotting of the relative amount of ATIII mRNA copies per cell versus the relative amount of ATIII cDNA copies per cell revealed a linear relationship between the relative amount of ATIII cDNA copies and the ATIII mRNA level for cell lines CHO-A11-A2, CHO-A11-A27, and CHO-A11-A279-C7 (regression coefficient = 0.996). The data from cell line CHO-A11-A279 did not fit into this linear relation (Figure 4).

Analysis of the translational level

The translational level was analyzed by comparing the relative amount of ATIII mRNA per cell with the amount of intracellular ATIII. The amount of intracellular ATIII of the four cell lines has been reported previously (Schröder and Friedl, 1997). When the relative amount of intracellular ATIII per cell was plotted against the relative amount of ATIII mRNA copies per cell (Figure 5) a linear relationship was observed that covered the whole range of expression level displayed by these four cell lines.

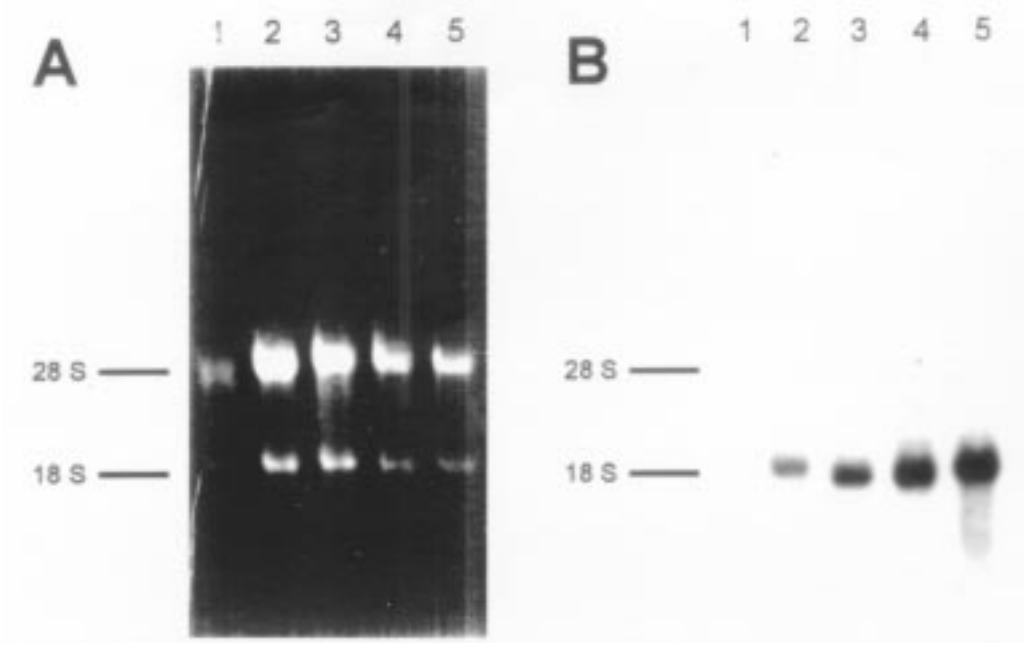

Figure 3. Northern blot analysis of the ATIII mRNA copy number in gene amplified CHO cells. (A) Formaldehyde agarose gel stained with ethidium bromide before capillary transfer took place. (B) Northern blot analysis of this gel with DIG-[11]-dUTP labeled ATIII cDNA prepared from plasmid pSVATIII as described under 'Materials and Methods'. 4 μg of total RNA from CHO DUKXB1 (lane 1), CHO-A11-A2 (lane 2), CHO-A11-A27 (lane 3), CHO-A11-A279 (lane 4), and CHO-A11-A279-C7 cells (lane 5) were loaded onto the gel.

Discussion

Much interest focuses on the production of therapeutic proteins in animal cells. Current techniques employing strong promoters or gene amplification are able to yield productivities of 20–40 $\mu g/10^6$ cells/24 h (Wood et al., 1990; Zang et al., 1995; Zettlmeissl et al., 1987). However, even these productivities are small when compared to other expression systems, e.g. bacteria (Hansson et al., 1994) and transgenic animals (Archibald et al., 1990). Thus, the engineering of high-producing animal cells is still a major research area.

It is of primary interest to localize the rate-limiting steps of gene expression at high expression levels. To achieve this goal we used a model system consisting of four CHO cell lines, which were engineered by gene amplification to express different amounts of ATIII (Zettlmeissl et al., 1987). ATIII was chosen as model protein, because of its relatively simple structure and its few posttranslational modifications (Franzén et al., 1980; Mizuochi et al., 1980). We have previously shown that an eightyfold increase in the amount of intracellular ATIII coindices with an only twentyfold increase in the specific ATIII-secretion rate and that misfolded ATIII is present in the highest amplified cell line. The secretion efficiency for ATIII dropped steadily as the expression level was elevated by gene amplification (Schröder and Friedl, 1997).

On the basis of a model for protein production in *E. coli* (Ryu et al., 1991) we have developed a model for the secretion of proteins by mammalian cells. According to this model linear relationships between the amount of ATIII cDNA copies, the ATIII mRNA level, and the amount of intracellular ATIII should exist as long as expression level does not influence transcription or translation efficiencies.

We have shown that a linear relationship between the amount of ATIII mRNA and the amount of intracellular ATIII exists. This linear relationship extends over the whole range of expression level covered by the model system (Figure 5) and up to the point where protein secretion becomes limiting (Schröder and Friedl, 1997). The relationship between the amount of ATIII cDNA and the amount of ATIII mRNA is more complicated, but overall the level of ATIII mRNA increased as ATIII cDNA copies were elevated (Figure 4). The increase in the amount of ATIII mRNA is proportional to the amount of ATIII

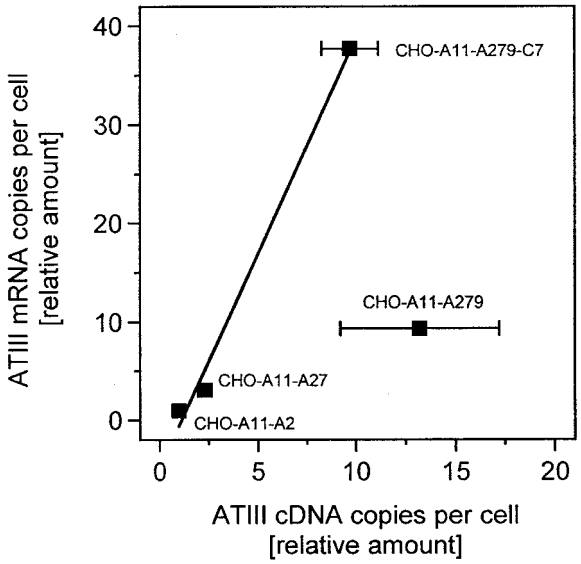

Figure 4. Plotting of the relative amount of ATIII mRNA copies per cell versus the relative amount of ATIII cDNA copies per cell. The line was calculated by linear regression and has a regression coefficient of 0.996.

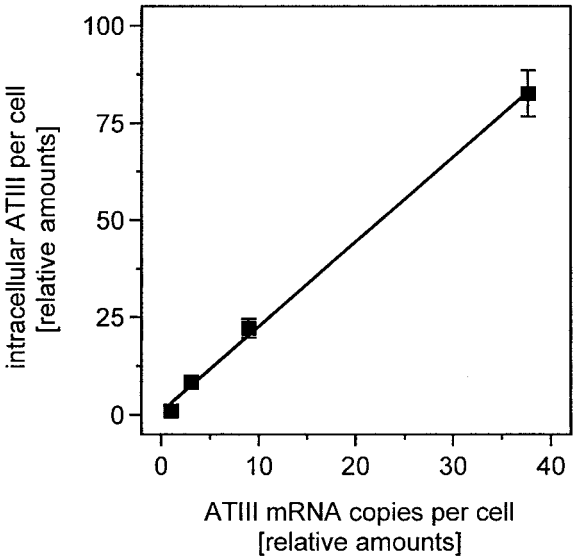

Figure 5. Plotting of the relative amount of intracellular ATIII per cell versus the relative amount of ATIII mRNA per cell. The line was calculated by linear regression and had a regression coefficient of 0.999. CHO-A11-A2 cells contained approximately 0.052 $\pm$ 0.003 μg ATIII per 10^6 cells (Schröder and Friedl, 1997).

cDNA genes in CHO-A11-A2, CHO-A11-A27, and CHO-A11-A279-C7 cells.

Ryu et al. (1991) included transcription and translation efficiencies in their model, which are useful tools to describe deviations from linearity. Our data does not allow the direct calculation of transcription or translation efficiencies, and it might be possible that they are far from one in all cell lines. From the linear relationship in Figure 4 and Equation (4) it can be concluded that all cell lines, except cell line CHO-A11-A279, possess approximately the same transcription efficiency. Transcription efficiency is not influenced by expression level up to ATIII secretion rates of 10 μg/10^6 cells/24 h. Therefore, the ATIII cDNA gene dosage present in CHO-A11-A279-C7 cells does not exceed the amount that can be efficiently transcribed by the cell. On the other hand it can be likewise ruled out that transcription of the ATIII cDNA genes is negatively affected by the decrease in secretion efficiency as expression level is elevated and the presence of misfolded ATIII in the highest amplified cell line.

The decreased transcription of the ATIII cDNA genes in CHO-A11-A279 cells (Figure 4) may be due to gene arrangements during the amplification process (Federspiel et al., 1984; Giulotto et al., 1986). Part of the copies might have come under the influence of genetic elements which downregulate transcription or have left regions which are under control of genetic elements which stimulate transcription (Wilson et al., 1990). Another possibility is that differences in the expression level or activity of transcription factors, factors involved in pre-mRNA splicing, nuclear export factors for mRNA or mRNA degrading enzymes contribute to the decreased transcription of ATIII cDNA genes in CHO-A11-A279 cells compared to the other three cell lines. However, a more general limited availability of these factors can be ruled out, because transcription of the ATIII cDNA genes in CHO-A11-A279-C7 cells is increased compared to CHO-A11-A279 cells. Furthermore cell line CHO-A11-A279-C7 is in a linear relationship with cell lines CHO-A11-A2 and CHO-A11-A27 (Figure 4), which possess only 1/10 or 1/4 of the ATIII cDNA copies of CHO-A11-A279-C7 cells, respectively. As the ATIII mRNA from CHO-A11-A279 cells has a comparable size distribution as ATIII mRNA from the other three cell lines (Figure 3B), a decreased stability of this mRNA in these cells seems unlikely, too.

A linear relationship between the amount of ATIII mRNA and intracellular ATIII was observed (Figure 5). This can be explained in two ways: I. Protein degradation is the major fate of intracellular ATIII. In this case the translation efficiency is equal for all cell lines and not influenced by expression level. To

Table 1. Calculation of ratios of successors to precursors for translation and secretion at different expression levels. The expression level increased from CHO-A11-A2 cells to CHO-A11-A279-C7 cells

Cell line	intracellular ATIII/ ATIII mRNA copies	(intracellular ATIII)^2/ ATIII mRNA copies	ATIII secretion rate/ intracellular ATIII
CHO-A11-A2	1.0	1.0	1.0
CHO-A11-A27	2.70±0.36	22.8±5.4	0.63±0.08
CHO-A11-A279	2.47±0.24	54.9±11.9	0.37±0.05
CHO-A11-A279-C7	2.19±0.15	181.0±26	0.25±0.02

discriminate between first- and second-order protein degradation the translation efficiency can be calculated from Equations (5). For first-order protein degradation the translation efficiency is proportional to the ratio $c_{protein,inside}/c_{mRNA}$, for second-order it is proportional to $c^2_{protein,inside}/c_{mRNA}$ (Table I). Our data supports first-order protein degradation in or near the secretory pathway. II. Secretion is the major fate of intracellular ATIII. In this case Equation (6) applies. To compensate for the drop in secretion efficiency a concomitant drop in translation efficiency has to occur. To estimate translation efficiencies on the basis of Equation (6) we calculated the ratio of successors to precursors for translation and secretion in the four different cell lines (Table I). With the aid of Equation (6) the change of the translation efficiency between the four cell lines may be estimated. The specific growth rate is small because the measurements were performed with stationary cultures and may be neglected, and the ratio of the rate coefficients k_3 to k_2 is by definition equal for all cell lines. The translation efficiency is proportional to the product of the ratio of intracellular ATIII to ATIII mRNA copies multiplied with the secretion efficiency (Equation (6)), which in turn is strictly proportional to the secretion frequency shown in Table I. Thus, the following values are derived for the four cell lines, which are proportional to the translation efficiency: 1.0 (by definition) for CHO-A11-A2 cells, 1.7 for CHO-A11-A27 cells, 0.9 for CHO-A11-A279, and 0.5 for CHO-A11-A279-C7 cells. In this case secretion and translation are tightly intertwined. Pathways for the regulation of translation when unfolded proteins are present in the ER have been described (Brostrom et al., 1996; Prostko et al., 1995; Srivastava et al., 1995). A general downregulation of translation seems unlikely, as the cells are still viable, and are easily grown in culture. The decrease in translation efficiency might explain at least to some part the 38-fold accumulation of ATIII mRNA in CHO-A11-A279-C7 cells compared to CHO-A11-A2 cells. mRNAs are stabilized in mammalian cells when either initiation or elongation of translation are inhibited with drugs (Ross, 1995). To discriminate whether protein degradation plays a major role in the secretory pathway or the downregulation of translation as unfolded proteins accumulate within the ER clearly further experiments have to be performed. However, the linear relationship between the amount of ATIII mRNA copies and intracellular ATIII rules out that the translational activity of the cells is exhausted at elevated expression levels. In this case deviations from linearity should be observed.

Conclusion

The basic conclusion to be drawn from this data for the engineering of mammalian cells with even higher productivities is that in any case the limiting step lies within the secretory pathway of mammalian cells. ATIII has a relatively simple structure with only three intramolecular disulfide bonds (Petersen et al., 1979), four biantennary *N*-linked oligosaccharides of the complex type (Franzén and Svensson, 1980, Mizuochi et al., 1980), and a molecular mass of 58 kDa (Nordenman et al., 1977). It is neither very large nor possesses posttranslational modifications that are observed only on a small number of glycoproteins. Thus, our observations should be of general significance and applicable to most attempts to express glycoproteins in CHO cells. Neither transcription or translation *per se* have been shown to be rate-limiting in our system of CHO cells, and the first activities that become rate-limiting are maturation and secretion of the recombinant protein. Translation may be downregulated as the secretion efficiency for ATIII drops or unfolded ATIII appears in the ER, but even in this case the point to intervene is at the secretory level. An

alternative is the engineering of a translational apparatus that is resistant to this downregulation, e.g. by mutating the phosphorylation site of PKR in eIF-2α (Murtha-Riel et al., 1993; Park et al., 1994; Ramaiah et al., 1994).

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