



## Competition between the signal sequence and a 3'UTR localisation signal during redirection of beta-globin mRNA to the endoplasmic reticulum: implications for biotechnology

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### Abstract

Secretion of an intracellular protein from a cell factory requires as a first step the redirection of the mRNA for synthesis of the protein on the endoplasmic reticulum. The feasibility of retargeting a mRNA coding for an intracellular protein to the endoplasmic reticulum was investigated using Ltk<sup>-</sup> fibroblasts stably transfected with gene constructs in which rabbit beta-globin coding region and 5'UTR was linked to albumin signal sequence and different 3'untranslated regions. Globin transcripts with the native globin 3'untranslated region or with the 3'untranslated region of c-myc are present in free/cytoskeletal-bound polysomes. The addition of the signal sequence from rat albumin redirects both these globin transcripts to membrane-bound polysomes but the presence of the c-myc 3'UTR reduces the extent of redirection. Globin transcripts with both the signal sequence and 3'untranslated region from the albumin gene are efficiently redirected to membrane-bound polysomes. The results suggest competition between 5' and 3' localising signals. The addition of the signal sequence does not destabilise the mRNA nor affect translational efficiency. It is concluded that it is possible to retarget an mRNA to the endoplasmic reticulum while maintaining stability and translational capacity. This has important implications for the development of vectors to promote secretion of intracellular proteins from cell factories.

### Introduction

Mammalian cell lines are increasingly being used to produce commercially significant amounts of recombinant proteins in cell factories. There are numerous examples where normally secreted proteins are being produced in this way (Bock et al., 1982; Michel et al., 1985; Lin et al., 1986; Devlin et al., 1987; Shak et al., 1990), as well as a number of commercial vectors for the secretion of heterologous proteins. Such systems have been almost exclusively used for the production of secreted proteins rather than proteins which are normally intracellular and the use of cell factories for commercial production of 'intracellular' proteins

is limited at present by the requirement to achieve secretion of the proteins into the culture medium. The development of technology to enable secretion of normally intracellular proteins, or a part of the appropriate molecule, would expand considerably the range of recombinant polypeptides which could be produced for biotechnological exploitation, for example for use in high-throughput screening. Secretion of intracellular proteins in mammalian expression systems is an increasingly important aim in biotechnological research (James and Simpson, 1996). To our knowledge there is only one paper describing the successful secretion of an intracellular protein from mammalian cells (Lee et al., 1996) but this protein (sialyltransferase),

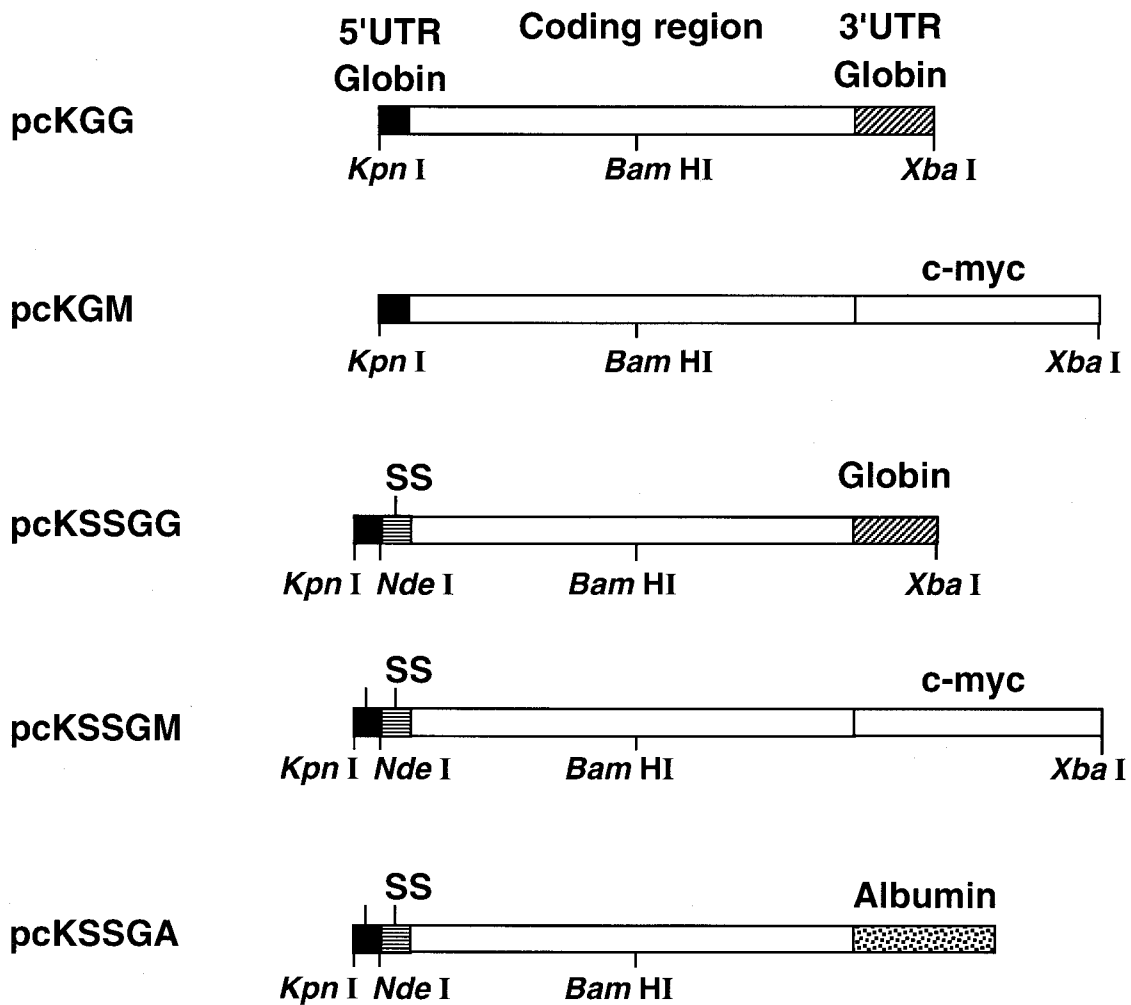


Figure 1. Schematic diagram of the constructs described in the Materials and methods. The parent vector is pcDNA3. The solid square represents the native 5'UTR of rabbit beta-globin. SS is the rat albumin signal sequence.

however, is normally synthesised on the rough endoplasmic reticulum (ER) and directed to the Golgi apparatus; successful secretion did not depend on an initial redirection of the mRNA for synthesis on the ER.

The secretion of a normally intracellular protein has two requirements: firstly, that there is a manipulation of the protein synthetic machinery so that the protein is made on the ER; secondly, that the protein passes through the secretory pathway without undue modification or degradation. The work described in this paper addresses the first stage in the process, namely the requirement that synthesis of the specific protein is retargeted to the ER since according to

present models of protein synthesis this is an absolute prerequisite for secretion.

It is well established that mRNAs coding for membrane and secreted proteins are translated on the ER in association with membrane-bound polysomes (Blobel and Dobberstein, 1975; Pryme et al., 1995). Secretory proteins contain a 16–30 amino-acid sequence at the N-terminal end and it is these sequences (signal sequence) in the nascent polypeptide chain which target the appropriate mRNA-ribosome complexes to the ER (Walter et al., 1984). A signal recognition particle (SRP) binds to the signal sequence and the SRP-mRNA-ribosome complex binds to a SRP-receptor in the ER; this initiates cotranslational transport of the protein through the ER membrane and synthe-

sis continues. In the absence of the ER interaction there is translational arrest. Secretory proteins pass through the membrane into the ER lumen and the signal sequence is then removed.

Recent work has shown that, in addition, there is an organisation of protein synthesis in the cytosol such that some mRNAs are localised in particular parts of the cytoplasm (Hesketh, 1966; Hovland et al., 1996; Bassell and Singer, 1997) and some are translated with polysomes associated with the cytoskeleton (CBP) rather than on free polysomes (FP). Thus, for example,  $\beta$ -actin mRNA is found in the cell periphery (Kislauskis et al., 1994) whilst c-myc and metallothionein-1 mRNAs are found in the perinuclear cytoplasm and in CBP (Hesketh et al., 1994; Mahon et al., 1997). It now appears that the protein synthetic apparatus is compartmentalised into at least three polysome populations – free (FP), cytoskeletal-bound (CBP) and membrane-bound (MBP) polysomes. In contrast to membrane and secreted proteins, whose synthesis is directed to the ER by the signal peptide, localisation of mRNAs in the cytoplasm or their association with the cytoskeleton involves signals in the 3' untranslated region (3'UTR) of the mRNA (Kislauskis et al., 1994; Hesketh et al., 1994; Wilson et al., 1995; Veyrune et al., 1996; Mahon et al., 1997). The precise nature and functioning of these signals has not been defined but they appear to involve RNA secondary structure and the binding of specific proteins to the 3'UTR (Hesketh, 1996; Ross et al., 1997).

The aim of the present work was to investigate whether a 3'UTR localisation signal would compete with a signal sequence in the retargeting of a mRNA coding for an intracellular protein from FP or CBP to the ER; to do this we used albumin signal sequence and c-myc 3'UTR as model signals. Since this retargeting is a prerequisite for ultimate secretion of the protein, information about signal competition is required in order to design efficient vectors for use in promoting the secretion of recombinant intracellular proteins.

## Materials and methods

### *Bacteria and cell culture*

Plasmids were cloned and propagated in *E. coli* strain DH5 (genotype *supE44 lacUI69 (80lacZ M15) hasR17 reAl endAl gyrA96 thi-1 relA1*).  $\text{Ltk}^-$  cells, a mouse fibroblast line, were grown in Dulbecco's minimal Eagle's medium supplemented with 10% foetal

calf serum. Cells were grown in an atmosphere of 5%  $\text{CO}_2$  in 90 mm Petri dishes for biochemical analysis and in 35 mm Petri dishes for transfection.

### *Vector construction*

All constructs (Figure 1) were derived from rabbit  $\beta$ -globin coding sequences by PCR cloning with the primers described below (underlined sequences are restriction sites and letters in lower case represent changes required to create restriction sites) and all contained the native globin 5'UTR. Each construct was verified by sequencing using an ABI 373A sequencer and cycle sequencing. The construction of pcKGG, a plasmid expressing rabbit beta-globin with native 5' and 3' UTRs has been described previously (Mahon et al., 1997). pcKGM expresses globin with the 3'UTR of mouse c-myc and was made as follows: the 3' half of the globin coding region and the entire c-myc 3'UTR was amplified by PCR using primers KP4 (GAC AGG CTG CAC GTG GAT CCT GAG AAC TTC) and KP5 (AAA AGA AAA CtC TaG aTG GCC CAA TTG TAT), KP4 maintaining a *Bam*HI site in the globin coding region and KP5 creating an *Xba*I site downstream of the c-myc poly-A signal, and plasmid MP13 as template (Veyrune et al., 1996). Using 1 ng template and 20 pmol of each primer in 1.5 mM  $\text{MgCl}_2$  and 4 Units Taq Polymerase (PerkinElmer), the reaction was cycled at 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 mins. The fragment and pcKGM were digested with *Bam*HI and *Kpn*I and ligated using standard methods (Sambrook et al., 1989).

pcKGA expresses globin 5'UTR and coding region linked to the 3'UTR of rat albumin mRNA. The 3'UTR was removed from the rat albumin gene by digestion with the restriction enzymes *Sal*I and *Bam*HI. The c-myc 3'UTR in pcKGM was then replaced by the albumin 3'UTR to give pcKGA: pcKGM was digested with *Xho*I and *Xba*I to remove the c-myc 3'UTR, blunt ends created by end-filling with Vent polymerase and the albumin 3'UTR inserted by ligation. Ligation and colony screening were carried out by standard methods (Sambrook et al., 1989).

Making the signal sequence constructs required creation of an appropriate restriction site at the AUG codon. This was accomplished by PCR using primers KP6 (GGA CTT TCC AAA ATC TCG TAA CAA CTC CGC) and KP7 (CTG GAC AGA TGC ACc atA TGG TCT GTT TTG); KP6 matches the sequence of pcDNA3 at position 731 and KP7 creates an *Nde*I site while maintaining the AUG and reading

frame. The template was pcKGG and the reaction was performed under the conditions described above. The product of the reaction was purified, and then 5  $\mu$ g was used in a PCR megaprime reaction with primer KP8-GAA GTT CTC AGG ATC CAC GTG CAG CTT GTC (covering a sequence in the globin coding region which includes a *Bam*HI site), 1  $\mu$ g pcKGG as the template and the cycles as described above. The resulting fragment was subcloned into pBluescript in the *Kpn*I and *Bam*HI sites, creating pBS-Nde.

The signal sequence was provided by annealing synthetic oligodeoxynucleotides that corresponded to the rat albumin signal sequence: SS1 – TAT GAA GTG GGT AAC CTT TCT CCT CCT CTT CAT CTC CGG TTC TGC CTT TTC and SS2 – TAG AAA AGG CAG AAC CGG AGA TGA AGA GGA GGA GGA GAA AGG TTA CCC ACT TCA. The insert was ligated into the *Nde*I site of pBS-Nde, creating pBS-SS. The *Kpn*I-*Bam*HI fragment from pBS-SS covering the 5' UTR, albumin signal sequence and globin coding region to the *Bam*HI site, was then ligated into appropriately digested pcKGG. pcKGM and pcKGA to create pcKSSGG pcKSSGM, and pcKSSGA.

#### *Transfection*

Transfections were carried out with LipofectAMINE™ (Gibco) according to the manufacturer's instructions. Cells were subcultured onto 35 mm Petri dishes at a density of  $5 \times 10^5$  cells/dish and grown to 70–80% confluence. The cells were overlaid with 1  $\mu$ g of plasmid DNA and 6–10  $\mu$ l LipofectAMINE in serum-free medium. After 5 hrs, serum was added to 10% final concentration. At 24 hrs the medium was replaced with complete medium and selection with 200  $\mu$ g/ml G418 was begun at 72 hrs. Cells were maintained in 100  $\mu$ g/ml G418 once stably transfected. The cell lines are named after the transfected construct i.e. the cells transfected with pcKGG is called GG, etc.

#### *Cell fractionation*

Ltk<sup>-</sup> cells were grown to 70% confluence, harvested using a rubber policeman and fractionated by a sequential detergent/salt extraction procedure (Vedeler et al., 1991; Hesketh et al., 1994). Polysomes were separated from monosomes and lighter ribonucleoprotein particles by centrifugation at 32,000 g for 17 hrs through a 15 ml cushion of 40% sucrose (Hovland et al., 1995).

#### *RNA Extraction and RNA gel electrophoresis*

Total RNA was extracted by the acid/guanidinium/phenolchloroform method of Chomczynski and Sacchi (1987), and the preparations were assessed by A<sub>260</sub>/A<sub>280</sub>. RNA was then separated by electrophoresis through a denaturing 2.2 M formaldehyde, 1.2% agarose gel (Sambrook et al., 1989) and transferred to nylon membrane (Genescreen, NEN Dupont, Ltd) by capillary blotting. RNA was fixed to the membrane by UV light and stored dry.

#### *Northern hybridisation and DNA probes*

Membranes were pre-hybridised at 42 °C for a minimum of 6 h with 0.1 mg/ml denatured salmon sperm DNA in 50% formamide, 10% dextran sulphate, 0.2% bovine serum albumin, 0.2% polyvinylpyrrolidone, 0.2% Ficoll, 0.1% sodium pyrophosphate, 1% SDS and 50 mM Tris HCl, pH 7.5. The beta-globin cDNA probe was described previously (Veyrune et al., 1996). The c-myc probe was a cDNA of the 3 exons of the murine gene (Hesketh et al., 1994) and was the gift of Dr. M. Cole (Princeton University, New Jersey, USA), the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was a 0.78 kb *Pst*I-*Xba*I fragment from the human cDNA (American Tissue Culture Collection, accession number 57090) and the 18S rRNA probe was a 1.4 kb *Bam*HI fragment from the cDNA (Erickson et al., 1981) obtained from Dr. R. Fulton, Beatson Institute, Glasgow. 20–30 ng of probe was labelled with <sup>32</sup>P dCTP by random priming (Megaprime kit from Amersham International UK). The labelled probe was added to the pre-hybridisation mix and hybridised overnight at 42 °C. The filters were washed briefly at room temperature in 2XSSC to remove hybridisation mix. Washes at 65 °C were probe specific as follows: globin-1X SSC 0.5% SDS 2X 30 min, c-myc-0.5X SSC, 1% SDS 2X 1 h, GAPDH-1X SSC, 1% SDS 2X 1 h, 18S-0.2X SSC, 1% SDS, 2X 1 h. A final brief wash in 0.1X SSC removed SDS. Specific hybridisation was detected and quantified on a Canberra Packard Instantimager. The amount of specifically bound probe was corrected for non-specific binding and the data expressed per unit of rRNA as measured by hybridisation to the 18S rRNA probe. Before being probed filters were stripped by heating them to 95 °C in 0.1% SDS for 10 min.

### mRNA stability

mRNA stability was determined by measuring abundance over a 2–12 h time period following the inhibition of transcription. Cells were grown to 70% confluence and transcription was inhibited by the addition of Actinomycin D (5  $\mu$ g/ml). RNA was extracted as described above.

### Polysome profiles and translational efficiency

Cells were grown to 70% confluency, scraped into the medium using a rubber policeman, then pelleted at 2000 rpm for 5 min at 4 °C. Cells were washed in DEPC PBS, and pelleted as before. The cells were resuspended in a solution of 10 mM TEA (triethanolamine) pH 7.6, 130 mM KCl, 5 mM MgCl<sub>2</sub>, 0.5 mM CaCl<sub>2</sub>, 0.25 mM sucrose containing 0.5% deoxycholate and 0.5% Triton X100, and incubated on ice for 10 min. The nuclei were pelleted by centrifuging at 3000 rpm for 10 min at 4 °C. 0.3 ml of the supernatant fluid was loaded onto a 10 ml 15%–40% sucrose gradient and centrifuged at 200,000  $\times$  g for 1 h. Gradients were monitored by measuring the absorbance at 260 nm using a flow-through cell mounted in a Zeiss PM 2A spectrophotometer and coupled to a W + W recorder. The outflow from the flow cell was collected and each gradient split into two fractions containing either polysomes or monosomes. Polysomes were pelleted by centrifuging at 32000  $\times$  g for 17 hrs and RNA was extracted from the pellet. Monosomal RNA was precipitated with isopropanol and ammonium acetate, then extracted as described earlier.

## Results

The experimental approach involved three stages: 1) the construction of a series of vectors with the albumin signal peptide and different 3' untranslated region (3'UTR) sequences linked to the rabbit beta-globin 5' untranslated region and coding sequence as a reporter gene; 2) the introduction of these constructs into cells by stable transfection and then 3) the determination of the subcellular location of the mRNA, its stability and translational efficiency. The experiments have been carried out using Ltk<sup>-</sup> fibroblasts, a cell line in which transfection of globin gene constructs and their localisation has been well characterised (e.g. Veyrone et al., 1996). mRNA association with the cytoskeleton and localisation through 3'UTR signaling

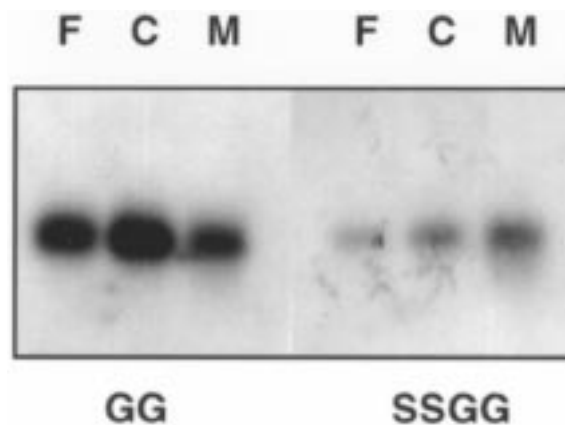


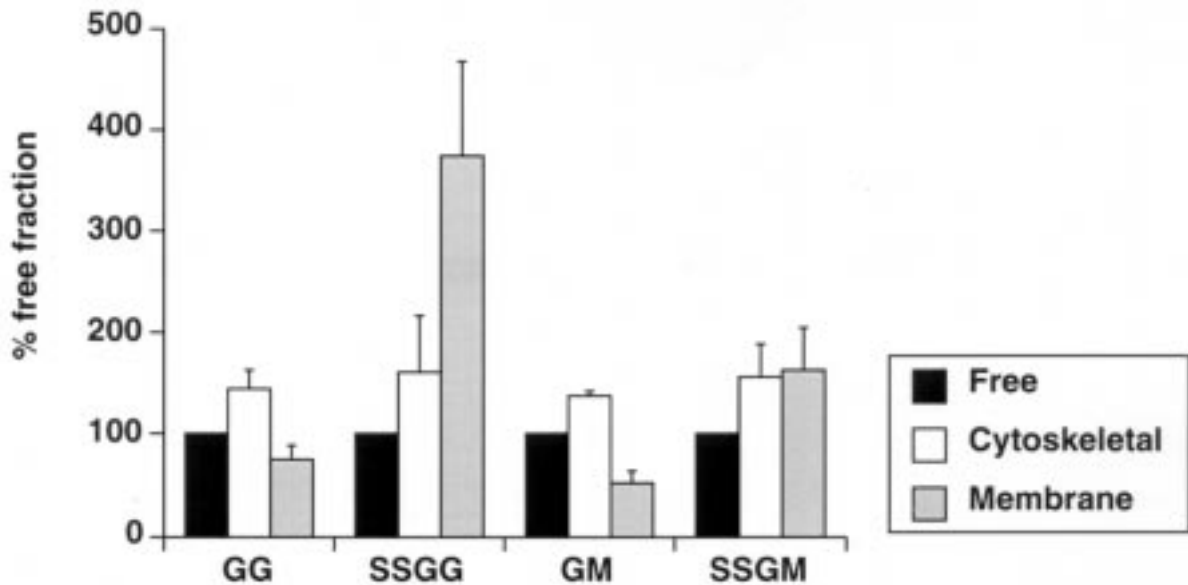
Figure 2. Northern hybridisation of total RNA from polysomes isolated from transfected cells. All lanes were loaded with 10  $\mu$ g RNA; the filter was hybridised with a rabbit beta-globin cDNA probe and specific bands detected by autoradiography at -70 °C for 3 days using Hyperfilm-MP. F, free polysomes; C, cytoskeletal-bound polysomes; M, membrane-bound polysomes.

appears to occur in a very wide range of cells (see Hesketh, 1996) and targeting to the ER by signal sequences is ubiquitous (see Walter et al., 1984); thus in principle, the findings in Ltk<sup>-</sup> cells are applicable to those lines, such as myeloma, CHO and BHK, which are used for commercial protein production.

### Redirection of globin mRNA by albumin signal sequence

Initially, four plasmids were constructed based on the rabbit beta-globin 5'UTR and coding region together with either the native globin 3'UTR or the c-myc 3'UTR and with or without the albumin signal sequence. These constructs were introduced into Ltk<sup>-</sup> fibroblasts and stable transfected cell lines were established. Expression of the transfected gene was assessed by Northern hybridisation to detect globin transcripts. All four genes were found to be expressed to the greatest extent when the cells were at 70% confluence (data not shown) and all subsequent experiments were carried out at that stage of growth.

Detergent/salt fractionation was performed to investigate the compartmentation of the transcripts between FP, CBP and MBP. Each cell line was fractionated, polysomes pelleted, and then RNA extracted from the pellets and analysed by Northern hybridisation for abundance of globin transcripts. Figure 2 shows that in contrast to the control cell lines (GG) where the globin transcript is found largely in FP/CBP, cells expressing the globin transcript with the albumin



**Figure 3.** Quantification of the distribution of globin mRNA in transfected cells. Results are expressed in arbitrary units mRNA per unit 18S rRNA obtained from direct radioactivity imaging using a Canberra Packard Instantimager. Abundance values were normalised by setting the abundance in free polysomes as 100 for each experiment. Results are means  $\pm$  S.E.M. ( $n=4$ ). Groups were compared using Student's 't' test: transcript abundance in the membrane-bound polysomes was significantly different between GG and SSGG cells ( $p=0.01$ ), GM and SSGM ( $p<0.05$ ) using a two-tailed test and between SSGM and SSGG using a one-tailed test.

signal sequence (SSGG) have the greatest abundance of this transcript in the MBP. Reprobing of filters with the 18S rRNA probe allowed correction for RNA loading and quantification of the hybridisation data per unit RNA. The abundance of globin transcripts in FP, CBP and MBP was respectively  $2.5\pm 0.4$ ,  $2.9\pm 1.0$  and  $2.1\pm 0.9$  (means from at least four separate experiments  $\pm$  s.e.m., expressed as arbitrary units of c.p.m. globin probe bound/c.p.m. 18S probe bound) in GG cells and  $0.9\pm 0.2$ ,  $1.3\pm 0.2$  and  $2.5\pm 0.8$  in SSGG cells. Since it was the relative transcript distribution rather than overall expression level that was the most important parameter in these experiments, these data were then expressed relative to the abundance in FP fractions; as shown in Figure 3 these data confirm that the addition of the albumin signal sequence redirects the globin mRNA to the ER ( $p=0.01$ ).

In the GM cells the abundance of globin transcripts in FP, CBP and MBP was respectively  $2.6\pm 0.7$ ,  $2.8\pm 0.9$  and  $1.6\pm 0.5$  (means from at least four separate experiments  $\pm$  s.e.m., expressed as arbitrary units of c.p.m. globin probe bound/c.p.m. 18S probe bound) and in SSGM cells the abundance distribution was  $2.5\pm 1.1$ ,  $2.3\pm 0.8$  and  $3.0\pm 1.4$ . Analysis of these data expressed as abundances relative to that in FP showed that the GM transcripts were present largely in CBP,

as found previously (Hesketh et al., 1994), but that, in contrast, the SSGM transcripts were found at similar relative abundances in CBP and MBP (Figure 3). The abundance of globin transcripts in MBP was significantly greater in SSGM cells than in GM cells, again showing the ability of the signal sequence to redirect transcripts to the ER. However, the increased abundance in MBP in SSGM cells compared to GM cells was significantly less ( $p<0.05$ ) than the increased abundance in SSGG cells compared to GG cells. Thus, although the signal sequence redirected the globin coding region and 5'UTR with the c-myc 3'UTR attached, the presence of both c-myc 3'UTR and the signal sequence reduced the extent to which redirection occurred. This suggests that there is competition between an ER localising signal (signal peptide) and a cytoskeletal localising signal (c-myc 3'UTR; Veyrune et al., 1996).

#### *Addition of the signal sequence has no effect on mRNA stability or translational efficiency*

Since it was theoretically possible that the manipulation of 5' and 3' signals may have altered the stability of the globin mRNA, we therefore determined the stability of the chimaeric transcripts. Each cell

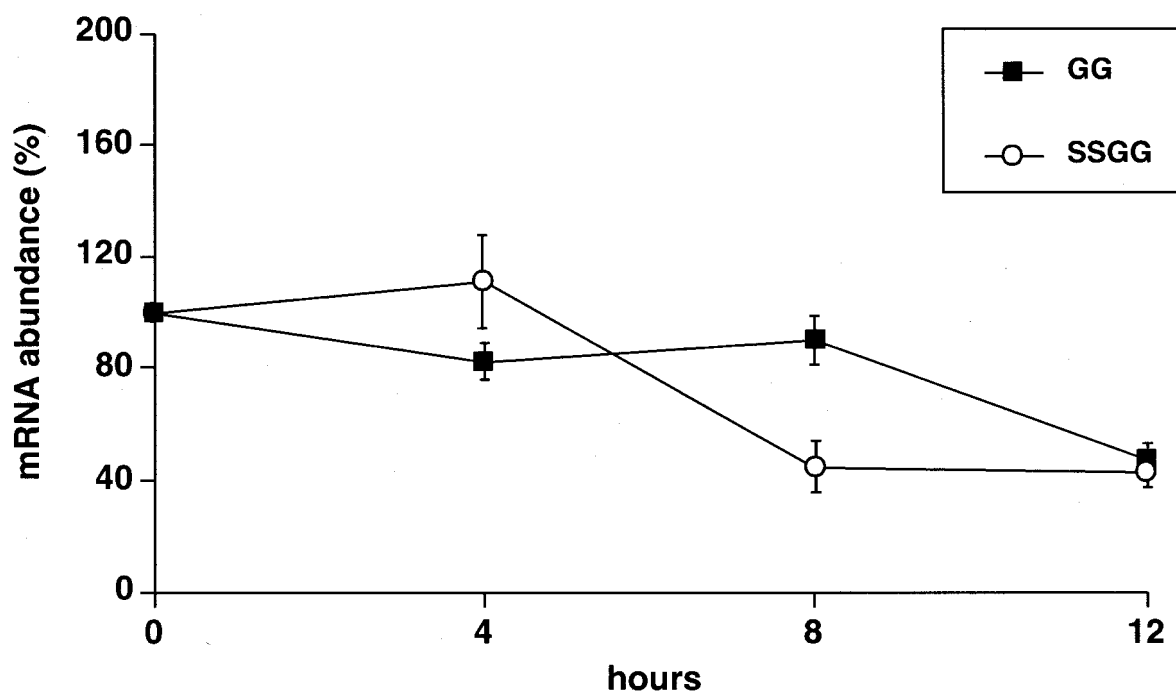


Figure 4. mRNA stability of the transcripts. GG and SSGG cells were grown to 70% confluence and transcription inhibited with Actinomycin D. RNA was extracted at specified time points and then analysed by Northern blotting. Quantification was carried out by direct radioactivity imaging using a Canberra Packard Instantimager. Results are means ( $n=6$ )  $\pm$  S.E.M. and are expressed as a percentage of initial abundance.

line was grown to 70% confluence and then treated with Actinomycin D to inhibit further transcription; RNA abundance was measured by Northern blotting. As globin is a highly stable message (Aviv et al., 1976) the abundances of GG and SSGG transcripts were measured over a 12 h period. As shown in Figure 4, the addition of the albumin signal sequence did not significantly alter the stability of the globin mRNA. Similarly, analysis of GM and SSGM cell lines showed that addition of the signal sequence to globin transcripts with the c-myc 3'UTR did not destabilise the mRNA (data not shown). Reprobing of the filters to detect GAPDH mRNA indicated that transcription had been inhibited in all four cell lines (data not shown).

In order to determine whether the manipulation of signals within the foreign gene had affected either translation in general or translation of the modified gene in particular, the protein synthetic apparatus of the four cell lines was subjected to polysome profile analysis. After cell lysis with salt and detergent, the soluble material containing total cell polysomes was loaded onto a 15%–40% sucrose gradient. Polysome profiles were recorded and are presented in Figure 5,

and polysome/monosome ratios in Table 1. Polysome profiles were very similar for all four lines with a similar proportion of ribosomes being present in active polysomes. The different peak heights represent differences in the total amount of RNA loaded onto the gradient. Thus, transfection with the four constructs did not affect overall protein synthesis. In addition, analysis of the proportion of globin transcripts in polysome and monosome fractions from the sucrose gradients (Table 2) shows that addition of the signal sequence did not significantly alter the translatability of the transcripts.

#### *The influence of the albumin 3'UTR*

The results in Figures 2 and 3 suggested that there is competition between 5' and 3' targeting signals but we were concerned that the observed effects could have been due to some form of non-specific interaction between the globin coding region and a heterologous 3'UTR which prevented the signal sequence performing its signalling role. To address this question we constructed a further vector with a different 3'UTR, namely the 3'UTR from the albumin gene: globin coding region with both the 3'UTR of rat albumin mRNA

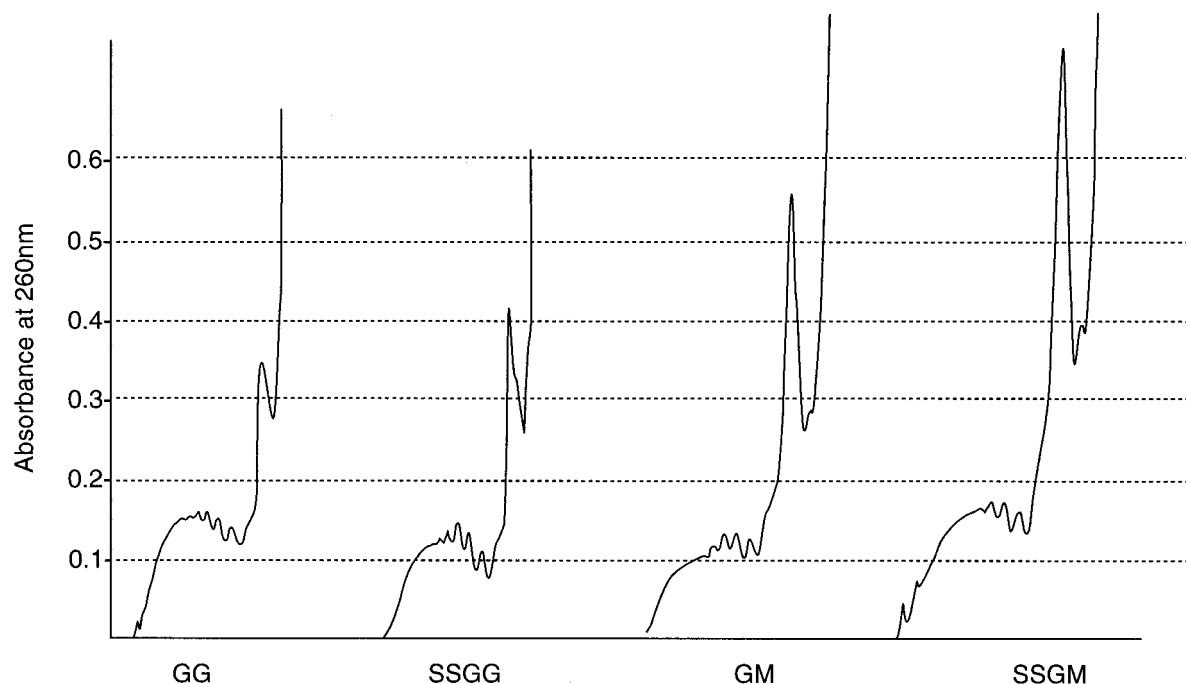


Figure 5. Polysome profiles of transfected  $Ltk^-$  cells. Soluble, detergent/salt extracted material containing the total cell polysomes was layered onto 15%–40% sucrose gradients and centrifuged at  $200\,000 \times g$  for 1 h. Polysome/ribosome distribution profiles were monitored by measuring the absorbance at 260 nm using a flow cell. Profiles are shown for the four cell lines GG, SSGG, GM and SSGM (as defined in the text) and in each case the top of the gradient containing the lighter material is at the right-hand side: a sharp peak of monoribosome material is present in each cell line followed by heavier polysome material.

Table 1. Distribution of ribosomes in polysomes and monosomes

Cell lines	Polysomes	Monosomes
GG	$56.5\% \pm 3.0$	$43.5\% \pm 3.0$
SSGG	$53.9\% \pm 1.7$	$46.1\% \pm 1.7$
GM	$51.7\% \pm 0.4$	$48.3\% \pm 0.4$
SSGM	$51.7\% \pm 1.1$	$48.3\% \pm 1.1$

Percentage distribution of ribosomes was calculated from the amounts of  $A_{260}$ -absorbing material present in polysomes and monosomes separated by sucrose density gradient centrifugation. Results are means  $\pm$  S.E.M. ( $n = 6$  for GG and SSGG,  $n = 9$  for GM and SSGM).

Table 2. Distribution of globin transcripts in polysomes and monosomes

Cell lines	Polysomes	Monosomes
GG	32.3%	67.7%
SSGG	42.1%	57.9%
GM	57.8%	42.2%
SSGM	48.5%	51.5%

RNA was extracted from polysome and monosome fractions recovered from sucrose density gradients (profiles shown in Figure 5) and analysed for globin mRNA abundance by Northern Blotting.

and the rat albumin signal sequence (SSGA). The plasmid was transfected into  $Ltk^-$  cells and stable cell lines established. The SSGA cells were fractionated and analysed as described above. The results in Figure 6 show that the SSGA transcripts containing both albumin 3'UTR and signal sequence were recovered predominantly in the MBP fraction. This indicates that introduction of a foreign 3'UTR is not sufficient, per

se, to prevent redirection of the globin transcript to the ER. A further vector (GA) was made in which the globin coding sequence was linked to the rat albumin 3'UTR but without the signal sequence; unfortunately, further analysis was not possible because we were unable to detect  $\beta$ -globin mRNA in the transfected cells.

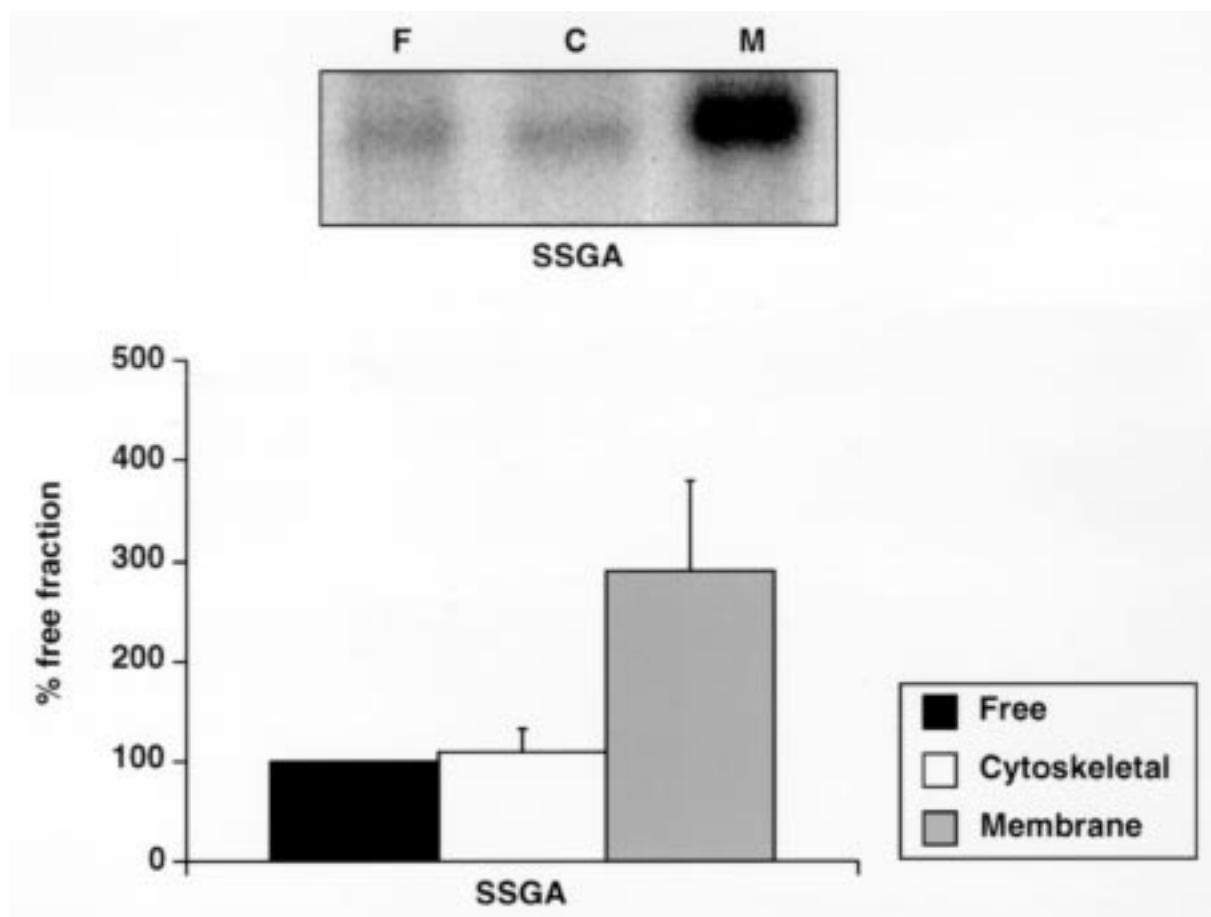


Figure 6. Globin transcript distribution in  $Ltk^{-}$  cells transfected with pcKSSGA. A Northern Hybridisation of RNA from free (F), cytoskeletal-bound (C) and membrane-bound (M) polysomes. All lanes were loaded with  $20 \mu\text{g}$  RNA. The filter was hybridised with a rabbit beta-globin cDNA probe. B. Quantification of mRNA abundance. Results are expressed in arbitrary units of mRNA abundance per unit 18S rRNA obtained from direct radioactivity imaging and normalised by setting the abundance in free polysomes as 100 for each experiment. Results are means  $\pm$  S.E.M. ( $n=3$ ).

## Discussion

The present results show that by a combination of DNA recombinant technology and transfection it is possible to produce stable cell lines in which globin transcripts are retargeted to the ER. Thus, in  $Ltk^{-}$  fibroblasts, the addition of the rat albumin signal sequence to globin mRNA with its native 3'UTR redirected the mRNA to MBP derived from the ER. This is the first demonstration of redirection of a mRNA from FP to the ER in a stably-transfected mammalian cell line. Redirection of histone mRNA to MBP has previously been demonstrated in mammalian cells (HeLa) using an *E. coli* signal sequence (Zambetti et al., 1987) but in this case the transfection was transient.

It is now clear that, in addition to mRNAs translated on FP, a considerable proportion of mRNAs are translated in polysomes associated with the cytoskeleton (Hesketh, 1996). The targeting of mRNAs to the cytoskeleton has been shown to be due to signals in the 3'UTR (Hesketh et al., 1994; Veyrune et al., 1996; Mahon et al., 1997). As shown in Figure 3, if in addition to a signal peptide, a cytoskeletal targeting signal is present in the 3'UTR of the transcript the shift to MBP is decreased. This appears not to be caused by a non-specific effect of a 'foreign' 3'UTR because addition of a different 'foreign' 3'UTR, that from albumin, to globin (SSGA; Figure 6) did not reduce the extent to redirection by the signal sequence. Thus, the c-myc 3'UTR localisation signal appears to interfere with mRNA sorting via a signal sequence and the data in-

dicates that when a hybrid transcript contains both a signal sequence and a 3'UTR localisation signal there is a competition between the 5' and 3' signals. Therefore, retargeting of a mRNA to the ER is less efficient in the presence of the 3'UTR localisation signal. In these experiments the c-myc 3'UTR has been used as a model localisation signal and it would be expected that similar competition would result from other such 3'UTR signals.

These data have important implications for biotechnology. They demonstrate that it is possible to create stable mammalian cell lines in which a mRNA coding for an intracellular protein is redirected to the ER, and this is the first step in facilitating the secretion of an intracellular protein. Although, Lee and co-workers (1996) have secreted active sialyltransferase from transiently transfected COS-7 cells using an IgM signal peptide, in this case the native sialyltransferase is synthesised on the ER and thus secretion did not require mRNA retargeting. Addition of a signal sequence to the green fluorescent protein has been found to promote secretion of this normally intracellular protein from insect cells (Laukkanen et al., 1996). Our results complement this work by demonstrating that addition of a signal sequence, in-frame and 5' to the initiation codon, retargets an intracellular mRNA to the ER. Furthermore the present data indicate that further modifications can be required to achieve efficient retargeting; if an mRNA coding for an intracellular protein contains a 3'UTR localisation signal then such a signal must be removed or inactivated in order to achieve efficient redirection of the mRNA. As indicated by the present results with the albumin 3'UTR (Figure 6), it may be most appropriate to use a vector containing a 3'UTR from the mRNA for a secreted protein, particularly one from the same gene as the signal sequence being used; in this way the desired coding region is inserted between the 5'UTR/signal sequence and 3'UTR from a mRNA for a secreted protein and the problem of signal competition is abolished.

If such modified genes are to be used for commercial purposes, ideally the signal modification should not decrease mRNA stability and translational efficiency to any major extent. The data in Figure 4 and 5 show that addition of the signal sequence to the globin coding region had no effect on mRNA stability or translational efficiency. Thus, retargeting an mRNA to the ER can be accompanied by a maintenance of mRNA stability and translational efficiency or capacity.

In conclusion, the present results show that it is possible to retarget a mRNA to the ER whilst maintaining stability and translatability. This is a prerequisite for secretion of an intracellular protein and the use of this technology in cell lines used for protein production has the potential to achieve secretion of recombinant intracellular proteins of biopharmaceutical interest from a cell factory. Development of the appropriate platform technology requires vectors in which any competing 3'UTR localisation signals are removed.

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