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Molecules and Cells

Identification of HUGT1 as a Potential BiP Activator and a Cellular Target for Improvement of Recombinant Protein Production Using a cDNA Screening System

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The development of a high-throughput functional genomic screening provides a novel and expeditious approach in identifying critical genes involved in specific biological processes. Here we describe a cell-based cDNA screening system to identify the transcription activators of BiP, an endoplasmic reticulum (ER) chaperone protein. BiP promoter contains the ER stress element which is commonly present in the genes involved in unfolded protein response (UPR) that regulates protein secretion in cells. Therefore, the positive regulators of BiP may also be utilized to improve the recombinant protein production through modulation of UPR. Four BiP activators, including human UDP-glucose:glycoprotein glucosyltransferase 1 (HUGT1), are identified by the cDNA screening. Overexpression of HUGT1 leads to a significant increase in the production of recombinant erythropoietin, interferon γ, and monoclonal antibody in HEK293 cells. Our results demonstrate that the cDNA screening for BiP activators may be effective to identify the novel BiP regulators and HUGT1 may serve as an ideal target gene for improving the recombinant protein production in mammalian cells.

INTRODUCTION

The human embryonic kidney cell line, HEK293, is one of the most commonly used human cell lines for large-scale production of recombinant biopharmaceuticals. With the development of the Epstein-Barr virus nuclear antigen-based HEK293 cell line that supports episomal replication of plasmids, large-scale transient gene expression (TGE) becomes a viable and convenient method for obtaining milligram quantities of recombinant proteins for research and analysis (Meissner et al., 2001; Wright et al., 2003). High-level expression of recombinant proteins, however, may be limited by the secretory capacity of the host cell. For example, our group previously showed that overexpression of the active spliced isoform of X-box binding protein 1 (XBP-1S) improved recombinant protein productivity in secretion-limited Chinese hamster ovary (CHO) cells (Ku et al., 2008). While a number of gene-targeting strategies have been devised to improve protein secretion in CHO cells (Borth et al., 2005; Davis et al., 2000; Ku et al., 2008), less has been done in HEK293. As such, secretion engineering in HEK293 cells may significantly benefit industrial TGE processes.

BiP (or GRP78), a widely used marker for endoplasmic reticulum (ER) stress, is a molecular chaperone localized in ER (Yoshida et al., 2001a). Its promoter contains an ER stress element (ERSE), which is responsible for transcription activation caused by unfolded protein response (UPR). As cellular UPR is induced by the accumulation of unfolded or malfolded proteins in the ER, secretory capacity of the cell is expanded through a coordinated up-regulation of chaperone genes, many of which are controlled by the ERSE (Yamamoto et al., 2004; Yoshida et al., 1998; 2001b). Therefore, improvement in protein secretion can be achieved by up-regulation of ER chaperones through selective induction of UPR. Recently, cDNA high throughput screening systems have been successfully applied to target the identification of specific biological processes or signaling pathways (Chanda et al., 2003; Huang et al., 2004; Liu et al., 2005). In this study, we performed a cDNA screening to identify BiP activators. One of the activators, human UDP-glucose: glycoprotein glucosyltransferase 1 (HUGT1), was found to improve transient production of recombinant erythropoietin (EPO), interferon γ (IFNγ), and monoclonal antibody in HEK293 cells.

MATERIALS AND METHODS

Plasmids and cDNA collection

The construction of cytomegalovirus (CMV)-EPO, CMV-IFNγ, CMV- green fluorescent protein (GFP), CMV-XBP-1S, CMV-XBP-1U plasmids, and heavy chain/light chain expression vectors for anti-Rhesus D (RhD) IgG has been previously de-

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Table 1. Fold induction of BiP-Luc vector caused by the respective genes. Results shown represent average and standard deviation of duplicate transfections. Genes causing ≥ 3-fold induction (highlighted) were used in follow-up studies.

Gene symbol	Fold induction		
pcDNA6[a]	0.96	±	0.13
pcDNA6	1.04	±	0.13
CEBPG	0.99	±	0.28
TDP1	0.64	±	0.01
CBR4	0.84	±	0.22
C1orf37	0.26	±	0.01
GPD2	0.52	±	0.21
XBP-1(h)	1.13	±	0.22
XBP-1(m)	1.08	±	0.22
BCL2	0.13	±	0.06
BCL6	0.46	±	0.06
TRAF2	1.38	±	0.62
P4HB	2.35	±	0.14
FKBP11	0.79	±	0.05
P58IPK[b]	**3.25**	±	**0.45**
P58IPK[c]	2.17	±	0.41
RAB27B	0.96	±	0.16
ERGIC53	0.96	±	0.09
HUGT1	**3.08**	±	**0.03**
BCAT1	0.83	±	0.09
SLC38A1	0.97	±	0.02
PPIA	0.84	±	0.05
TRA1	**7.26**	±	**0.59**
CDK7	0.87	±	0.07
Cyclin A1	0.73	±	0.02
Cyclin K	1.20	±	0.07
CDKN1B	0.44	±	0.14
CDKN3	0.64	±	0.00
HDAC4	0.33	±	0.04
HDAC5	0.56	±	0.11
HDAC8	0.84	±	0.10
HDAC11	0.42	±	0.03
SIRT1	0.66	±	0.01
HOXB4	1.54	±	0.17
PTX1	1.30	±	0.07
Msx2	0.82	±	0.00
CDK1	1.17	±	0.08
CDK9	0.72	±	0.06
CDK9 D167N	0.69	±	0.15
HMGI-C	1.15	±	0.05
HMGI	0.88	±	0.01
HMGY	0.71	±	0.08
PBX1a	1.76	±	0.14
PBX1b	0.45	±	0.04
MEIS1	1.22	±	0.04
PREP1	1.27	±	0.22
PDX1	2.27	±	0.13
HOXA9	1.55	±	0.08
CDP	0.85	±	0.11
PIN1	1.68	±	0.32
HIV Tat	0.58	±	0.04
QSCN6	0.86	±	0.17
BOK	0.91	±	0.17
FLJ10979	1.36	±	0.37
BAK1	0.18	±	0.04
HEYL	0.88	±	0.02
CHOP	1.15	±	0.11
ALG12	2.12	±	0.02
HEXIM1	0.56	±	0.01
HEXIM2	1.04	±	0.17
FLJ10979	1.21	±	0.03
COG1	**4.47**	±	**0.89**
PRSS12	1.24	±	0.04
WBP5	1.34	±	0.03
NF-YC	2.15	±	0.17
GADD45G	2.26	±	0.32
CGI-10G	0.99	±	0.17
PSAT1	1.71	±	0.06
SLC3A2	0.82	±	0.17
PPIB	0.92	±	0.31
CDK6	1.09	±	0.18
Cyclin D1	1.32	±	0.38
CDKN2A	1.34	±	0.32
CDKN2C	1.10	±	0.24
CDKN2D	1.38	±	0.30
SIRT3	1.81	±	0.54
ATF3	1.53	±	0.70
SIRT2	0.73	±	0.15
XBP-1S (m)[d]	15.14	±	3.52
XBP-1U (m)[e]	1.50	±	0.31

[a]Empty pcDNA6 plasmids were used as negative controls.
[b]Short isoform of P58IPK
[c]Long isoform of P58IPK
[d]Mouse XBP-1S was used as a positive control.
[e]Mutated mouse *xbp-1* mRNA to express only XBP-1U

scribed (Chusainow et al., 2009; Ku et al., 2008). The BiP-Luc plasmid was kindly provided by Dr. Kazutoshi Mori (Yoshida et al., 2000). The cDNA collection consists of 81 human/mouse gene sequences and a human immunodeficiency virus (HIV) gene, Tat (Table 1). Expression plasmids of our cDNA collection were either purchased (Open Biosystems) or cloned in-house. Expression of all cDNAs was driven by the human CMV major immediate-early promoter. The library screen was conducted in a 96-well format.

Cells, transient transfection and luciferase assay

HEK293 cells were obtained from American Type Culture Collection (ATCC). Transient transfections were performed using FuGENE 6 Transfection Reagent (Roche) according to manu-

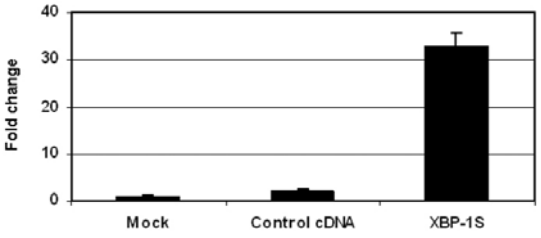

Fig. 1. Assay development for cDNA screening. 20,000 HEK293 cells were plated into each well of a 96-well plate one day prior to transfection. After overnight incubation, cells were separately co-transfected with 0.1 μg of BiP-Luc and 0.1 μg of the indicated expression plasmid [i.e., pcDNA6 (Mock), CMV-HEXIM2 (HEXIM2), and CMV-mXBP-1S (XBP-1S), respectively]. Transient transfections were performed using FuGENE 6 Transfection Reagent (Roche) according to manufacturer's protocol. Luciferase activity was assayed two days post-transfection. The assay was repeated and similar results were obtained. Representative results from one transfection are shown.

facturer's protocol. For cDNA screening, cells were transiently co-transfected with the reporter vector, BiP-Luc, and one of the expression plasmids from our cDNA collection. Luciferase activity was assayed 2-day post-transfection using the Bright-Glo™ Luciferase Assay System (Promega). Luciferase signals were measured using the Genios microplate reader (Tecan).

Enzyme-linked immunosorbent assay (ELISA) and western blotting

HEK293 cells were co-transfected with the indicated expression vector (i.e., CMV-EPO, CMV-IFNγ, or heavy chain/light chain expression plasmids for anti-RhD IgG) and a plasmid encoding a candidate gene identified from the BiP-Luc cDNA screening. Culture supernatants were collected two days post-transfection and analyzed by ELISA or Western blotting. Amounts of secreted EPO and IFNγ in culture supernatant were quantified using EPO (Roche) and IFNγ (HyCult biotechnology) ELISA kits, respectively. The concentration of secreted human anti-RhD IgG in the transfected culture supernatants was measured using sandwich ELISA as previously described (Chusainow et al., 2009). Western blots were carried out using the standard protocol. To examine the completely assembled anti-RhD antibody, the protein samples were analyzed on a non-reducing protein gel followed by Western blot using anti-human IgG (H + L) antibody (Jackson ImmunoResearch). Anti-EPO (ATCC), anti-IFNγ (Santa Cruz Biotechnology), and purified human IgG1-κ (Sigma) antibodies were also purchased for the respective blots.

RESULTS AND DISCUSSION

Identification of four BiP activators by BiP-Luc cDNA screening

Before screening our cDNA panel for the BiP activators, we first worked on the pre-screening assay development to optimize the experimental conditions. A luciferase-expressing plasmid, BiP-Luc, was used as a reporter, in which the expression of luciferase is driven by BiP promoter. Under the optimized condition, HEK293 cells were co-transfected with BiP-Luc and an indicated vector [i.e., either blank vector, mXBP-1S, or hexamethylene bis-acetamide inducible 2 (HEXIM2) expression plasmids] (Fig. 1). The mock plasmid was used as a negative control. The expression vector of XBP-1S, which was previously demonstrated as a potent activator of BiP-Luc (Ku et al., 2008), was used as a positive control. HEXIM2 was randomly picked from our cDNA collection. Results showed that cells overexpressing HEXIM2 displayed a 1.2-fold induction of luciferase driven by BiP promoter (Fig. 1). In comparison, the positive control, mXBP-1S, resulted in a 33-fold increase in expression of the luciferase (Fig. 1). The significant difference between luciferase readouts of the positive and negative controls demonstrated the sensitivity and reliability of the optimized BiP-Luc assay to identify the potential BiP activators.

The BiP-Luc screen was then conducted using our cDNA panel. This cDNA panel consists of genes known to be involved in transcription, UPR, cell cycle regulation, as well as those identified through transcription profiling in batch and fed-batch bioreactor cultures of HEK293 cells (Lee et al., 2007) (Table 1). Individual cDNA plasmids along with the BiP-Luc reporter were co-transfected into HEK293 cells growing in a 96-well plate. Duplicate transfection was performed and luciferase activity was assayed two days post-transfection. Genes producing an average induction of more than three fold were classified as the potential BiP activators or candidate genes for follow-up studies. Four candidates, COG1 (component of oligomeric Golgi complex 1), HUGT1, TRA1 (tumor rejection antigen 1), and P58IPK [DnaJ (Hsp40) homolog, subfamily C, member 3], were identified based on the preset criterion. The functions of the genes are summarized in Table 2. While it is not surprising that all the four genes are involved in secretory pathway, the precise mechanisms by which these genes activate the BiP promoter remain to be determined. As these genes are not known to function as transcrip-

Table 2. Details of the candidate genes identified by BiP-Luc screening

Symbol	Accession	Gene name	Function
P58IPK	NM_006260	Homo sapiens DnaJ (Hsp40) homolog, subfamily C, member 3 (DNAJC3)	Part of a negative feedback loop in unfolded protein response to inhibit the phosphorylation of eIF2α (van Huizen et al., 2003). Co-chaperone mediating cotranslational ER protein degradation (Oyadomari et al., 2006)
HUGT1	NM_020120	Homo sapiens UDP-glucose:glycoprotein glucosyltransferase 1[a], transcript variant 1	Reglucosylates unfolded proteins in the ER (Arnold et al., 2000)
TRA1	NM_003299	Homo sapiens tumor rejection antigen 1 (TRA1)[b]	Stress-inducible ER chaperone (Sorger and Pelham, 1987)
COG1	NM_018714	Homo sapiens component of oligomeric Golgi complex 1 (COG1)	One of eight proteins which form a Golgi-localized complex required for Golgi morphology and function (Ungar et al., 2002)

[a]Also known as: UDP-glucose ceramide glucosyltransferase-like 1 (UGCGL1)
[b]Also known as: heat shock protein 90kDa beta (Grp94), member 1 (HSP90B1)

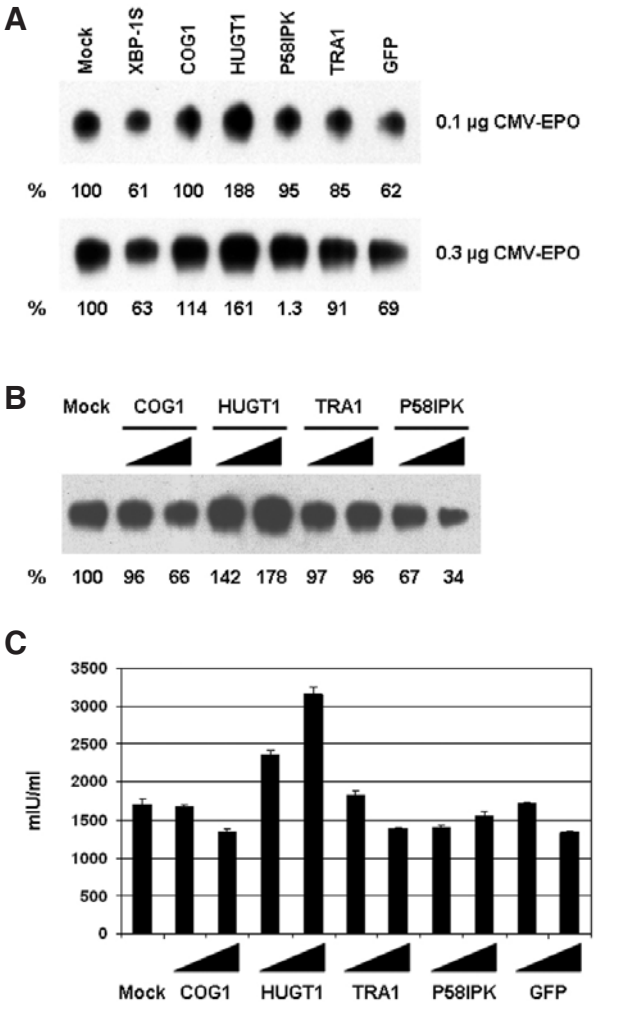

Fig. 2. Effects of the candidate genes on transient EPO production. (A) HEK293 cells were transiently co-transfected with 0.1 or 0.3 μg CMV-EPO vector and 0.2 μg of the indicated expression plasmid to overexpress XBP-1S, GFP, COG1, HUGT1, TRA1, or P58IPK. EPO content in culture supernatant was analyzed by Western blotting two days post-transfection. Western blots were carried out followed by the standard protocol using a mouse monoclonal anti-EPO antibody purified from culture supernatant of a hybridoma HB-8209 (American Type Culture Collection). Integrated intensity of each protein band in Western blotting was quantified by a densitometer (Bio-Rad) and normalized to that of mock transfection. (B, C) HEK293 cells were transiently co-transfected with 0.1 μg CMV-EPO vector and 0.1 or 0.3 μg of the indicated expression plasmid. EPO titers were analyzed by Western blotting (B) or ELISA (C) two days post-transfection. ELISA was performed using EPO ELISA kit (Roche) according to manufacturer's protocol. The experiment was repeated and similar results were obtained. Representative results from one transfection are shown.

tion activators in the secretory ER stress pathways, it may be possible that overexpression of these proteins may be perceived by the host cell as stress induction, triggering ER stress-related transcription activation. Regardless of the precise mechanism, the usefulness of these target genes for improving recombinant protein production needs to be verified.

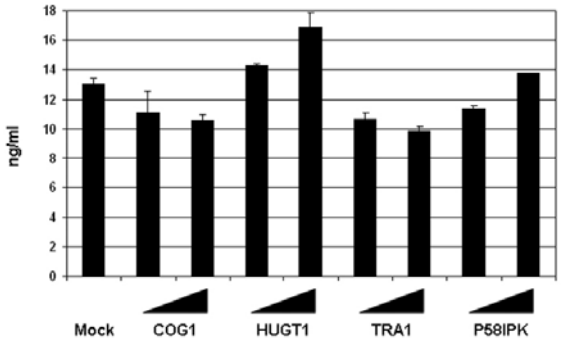

Fig. 3. Effects of the candidate genes on transient IFN-γ production. HEK293 cells were transiently co-transfected with 0.1 μg CMV-IFN-γ plasmid and 0.1 or 0.3 μg of the indicated expression vector (i.e., COG1, HUGT1, TRA1, and P58IPK). IFN-γ content in culture supernatant was analyzed by IFN-γ ELISA kit (HyCult biotechnology) two days post-transfection. The experiment was repeated and similar results were obtained. Representative data from one transfection is shown.

Improvement of recombinant protein production in HEK293 cells by HUGT1

The effects of the four BiP activators on production of recombinant biopharmaceutical proteins, including EPO, IFNγ, and anti-RhD IgG were examined. HEK293 cells were transiently co-transfected with an EPO expression vector (i.e., CMV-EPO) and an indicated plasmid encoding one of the BiP activators. First, different dosages of the EPO plasmids (i.e., 0.1 and 0.3 μg) were included in the assays. Culture supernatant was sampled two days post-transfection and analyzed by Western blotting (Fig. 2A). Besides the empty vector control (Fig. 2A, "Mock"), two additional controls, XBP-1S and GFP, were also included. No activating effects were observed in XBP-1S or GFP (Fig. 2A). XBP-1S was a BiP activator while GFP served as a non-specific control. Since we previously showed that XBP-1S only improved recombinant protein production in secretion-limited producing cells (Ku et al., 2008), the results presented here suggested that protein secretion was not the bottleneck for these transient EPO-expressing HEK293 cells. Up to 88% increases in EPO titers were detected in HUGT1-expressing cells, but not in COG1-, TRA1-, and P58IPK-transfected cells (Fig. 2A). A similar set of experiments were performed by titrating the amounts of expression plasmids (i.e., 0.1 and 0.3 μg) for the selected genes. Culture supernatant was collected two days post-transfection and analyzed by Western blotting and ELISA. Only HUGT1 consistently enhanced EPO production under both conditions (Fig. 2). About 80% increase in EPO production was detected in the HUGT1 expressing cells transfected with high dose of the expression plasmid (Figs. 2B and 2C).

Effects of the four candidate genes on transient IFNγ production were examined next. A lower increase (up to 30%) in IFNγ titers was detected when HUGT1 was overexpression, while no significant improvement was detected in the COG1, TRA1, and P58IPK overexpressing cells (Fig. 3).

Overexpression of HUGT1 also significantly improved the transient production of anti-RhD antibody. Samples were collected three days post-transfection and analyzed by ELISA. Up to 3-fold increase in antibody titers was detected, while little or no effects were observed in the COG1, TRA1, or P58IPK overexpressing cells (Fig. 4A). An anti-Fc specific antibody was used in the antibody ELISA. Therefore, the ELISA results may not truthfully reflect the quantities of completely assembled anti-

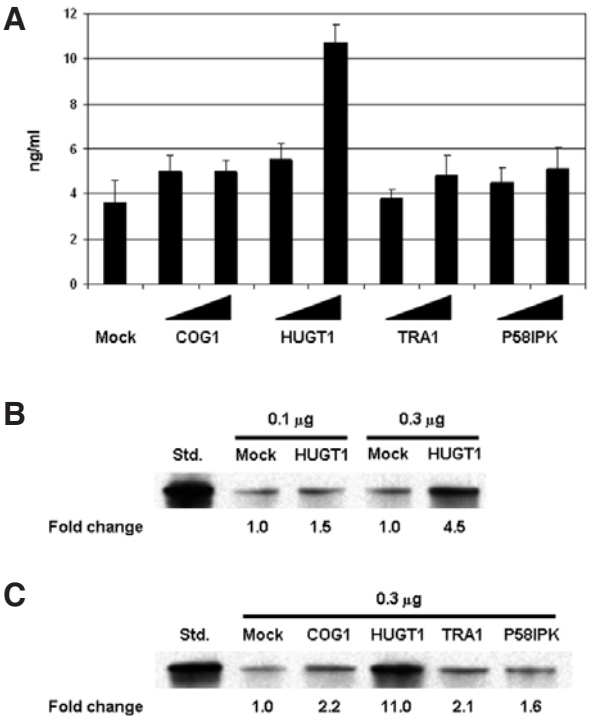

Fig. 4. Effects of the candidate genes on antibody production. (A) HEK293 cells were transiently co-transfected with 0.1 or 0.3 μg of the indicated expression vector (i.e., COG1, HUGT1, TRA1, and P58IPK) and 0.1 μg plasmid mixture to express anti-RhD IgG (i.e., 0.07 and 0.03 μg light chain and heavy chain expression plasmids, respectively). Antibody titer in culture supernatant was analyzed by ELISA three days post-transfection. (B, C) Generation of completely assembled antibody was analyzed on a non-reducing protein gel. Samples were collected three days post-transfection. The purified human IgG1-κ antibody (Sigma) was used as a standard (i.e., Std.). Integrated intensity of each protein band in western blotting was quantified by a densitometer (Bio-Rad) and normalized to that of mock transfection. These experiments were repeated and similar results were obtained. Representative data from one transfection is shown.

RhD IgG. Non-reducing SDS-PAGE was carried out followed by Western blotting and a purified human IgG1-κ antibody was used as a standard for the properly assembled antibody (Figs. 4B and 4C, Std.) Results showed that HUGT1 overexpression led to more than 4-fold improvement in the production of completely assembled antibodies (Figs. 4B and 4C). Collectively, HUGT1 can enhance all three biopharmaceuticals, especially recombinant antibody, examined in this study (Figs. 2-4).

Like HUGT1, COG1, TRA1, and P58IPK were identified as potential BiP activators in our screening by activating the expression of firefly luciferase driven by the BiP promoter (Table 1). However, little or no detectable effects were observed when COG1, TRA1, or P58IPK were overexpressed (Figs. 2-4). Firefly luciferase, which is not a secreted protein, was used as the reporter in our BiP-Luc assay. Therefore, an increase in luciferase expression might not guarantee the parallel improvement of secreted recombinant proteins, such as EPO, IFNγ, or antibody. In addition, since UPR signaling involves a delicate balance between expanding the secretory capacity, shutdown of protein synthesis, enhanced protein degradation, and onset of apoptosis, overstimulation of the BiP promoter may signify a triggering of pathways unfavorable for overall protein productivity. Similar observations have been made when overexpression of BiP protein resulted in intracellular retention, rather than enhanced secretion, of monoclonal antibody in CHO cells (Borth et al., 2005).

In conclusion, using this BiP-Luc cDNA screening, we have successfully identified four potential BiP activators and one of them, HUGT1, may be utilized to improve production of EPO, IFNγ, and therapeutic antibody at least in HEK293 cells. An effective functional genomic screen therefore provides a rapid way of identifying the potential useful BiP activators, facilitating the development of cell line engineering strategies that are specific for different recombinant protein production systems.

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