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Production of Recombinant Proteins

Challenges and Solutions

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Summary

Efficient strategies for the production of recombinant proteins are gaining increasing importance, as more applications that require high amounts of high-quality proteins reach the market. Higher production efficiencies and, consequently, lower costs of the final product are needed for obtaining a commercially viable process. In this chapter, common problems in recombinant protein production are reviewed and strategies for their solution are discussed. Such strategies include molecular biology techniques, as well as manipulation of the culture environment. Finally, specific problems relevant to different hosts are discussed (*see* Chapters 1 and 3).

Key Words: Fermentation; prokaryotes; yeasts; fungi; animal cells.

1. Common Problems Encountered During Production of Recombinant Proteins

The demand of recombinant proteins has increased as more applications in several fields become a commercial reality. Recombinant proteins have been utilized as tools for cellular and molecular biology. Various application areas have experienced substantial advances thanks to the possibility of producing large amounts of recombinant proteins by an increasing availability of genetically manipulated organisms. For instance, uncountable lives have been saved because of the almost unlimited accessibility of therapeutic and prophylactic proteins that before the era of modern biotechnology could be obtained only in very small amounts from unsafe sources. Today, more than 75 recombinant proteins are utilized as pharmaceuticals, and more

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than 360 new medicines based on recombinant proteins are under development (www.phrma.org). The impact of the production of recombinant proteins has also extended to the development of bioinsecticides, diagnostic kits, enzymes with numerous applications, and bioremediation processes, among many others. In particular, areas such as detergent production and food processing have been among the most notable success.

Even when hundreds of proteins are produced at commercial scale, the production of recombinant proteins still constitutes a challenge in many cases. Moreover, many applications would benefit with higher production efficiencies and consequent lower costs of the final product. In this chapter, typical problems encountered during recombinant protein production are reviewed and strategies to solve them and increase productivity are discussed.

1.1. Loss of Expression

A necessary condition for adequate recombinant protein production is the efficient expression of the gene of interest. However, expression can be lost due to structural changes in the recombinant gene or disappearance of the gene from host cells. Loss of expression will be discussed here, with emphasis on the three alternative locations of the gene of interest: in plasmids, integrated to the host's chromosome, or delivered by a virus.

1.1.1. Plasmid-Based Systems

Plasmids are extrachromosomal self-replicating cytoplasmic DNA elements that are found in prokaryotes and eukaryotes. They have been used as molecular vehicles for recombinant genes since the dawn of genetic engineering. Plasmid-based expression is the most popular choice when using prokaryotes as hosts, as genetic manipulation of plasmids is easy. Furthermore, gene dose, which depends on plasmid copy number, is higher than when the recombinant gene is integrated into the host's chromosome. Plasmid copy number is an inherent property of each expression system and depends on the plasmid, the host, and the culture conditions (***1***). In particular, plasmid copy-number is regulated by copy-number control genes (***2***). Plasmid copy number can range from a few up to 200. Plasmids impose a metabolic load on the host, as cellular resources must be utilized for their replication as well as for the expression of plasmid-encoded genes and production of recombinant protein. The metabolic load increases with an increase in the size of the insert, temperature, expression level, recombinant protein yield, and toxicity of the expressed protein toward the host (***3,4***). Such a metabolic load often results in a decrease in the growth rate of plasmid-bearing cells. As copy number increases, the metabolic load increases. Consequently, growth rate decreases (***2***) and faster-growing plasmid-free cells eventually overtake the culture.

Plasmid loss is the main cause of reduced recombinant protein productivity in plasmid-based systems. An unequal plasmid distribution upon cell division will eventually lead to plasmid-free cells. This is called plasmid segregational instability. Plasmid copy number depends on the number of plasmid copies at the time of cell division and their random distribution between daughter cells (***2***). If plasmid number is high (>10),

the probability that a plasmid-free daughter cell will emerge is extremely low (***4***). Another factor that increases plasmid instability is plasmid multimerization. As plasmid copies have the same sequence, they can recombine and form a single dimeric circle with two origins of replication. This results in fewer independent units to be segregated between daughter cells, and consequently plasmid loss can increase (***4***). In addition, cells bearing multimers grow more slowly than those bearing monomers, even at the same copy numbers (***4***). Other parameters that influence plasmid stability are plasmid size (larger plasmids are less stable) (***5***), the presence of foreign DNA (***3***), cell growth rate, nutrient availability, temperature, and mode of culture, which will be further discussed in **Subheading 2**.

Several natural mechanisms exist to ensure plasmid survival in cell populations (***6***). For example, low-copy-number plasmids guarantee their persistence by multimer resolution through site-specific recombination systems (*cer* sequence) or active partition mechanisms, such as the *par* sequences (***2***). Genes responsible for both mechanisms have been incorporated in man-made plasmids to increase their stability (***7***). Plasmid instability is prevented if plasmid-bearing cells have a competitive advantage over plasmid-free cells. Thus, selective pressure can be utilized to select for plasmid-containing cells. The strategy most commonly used is to introduce into the plasmid a gene or genes that provide resistance to particular antibiotics. Selective pressure is then applied by supplementing the antibiotic to the culture medium. This approach can be ineffective if antibiotics are degraded or inactivated, or if periplasmic detoxifying enzymes leak from plasmid-containing cells (***5***). Moreover, antibiotics are expensive, and their presence is undesirable in food and therapeutic products as well as in the exhausted culture broth that is discharged to wastewater treatment facilities of large-scale fermentation operations. Accordingly, other forms of selective pressure have been explored, such as deletion of an essential gene from the bacterial chromosome and its inclusion in the plasmid, or the introduction of a growth repressor in the bacterial genome and its antidote in the plasmid (***8***).

Plasmid structural instability is another form in which foreign gene expression can be lost (***1***). In this case a genetic reorganization of the plasmid structure occurs, yielding a nonproductive vector (***3***). Structural instability is less common than segregational instability and cannot be prevented through selective pressure. On the contrary, strong positive selection at the time of foreign gene expression can induce structural instability (***3***). Structural instability can result either in a complete elimination of recombinant protein production or in the accumulation of aberrant recombinant proteins with minor changes in the original amino acid sequence (deletions, additions, or substitutions). The latter situation can be even more insidious than the former because its presence is usually not evident, as selection markers can remain unchanged. Thus, complete amino acid sequencing of the recombinant protein or DNA sequencing of the gene of interest must be performed to detect such a problem.

Another important issue in plasmid-based systems is plasmid copy number. Although high plasmid copy numbers are generally desired for improving recombinant protein yield, this might not always be true. For instance, high copy numbers may drive high protein production rates, which can result in protein aggregation and deficient

posttranslational modification (***8***). Low recombinant protein yields can also occur in cells with a high plasmid copy number, possibly because of a reduction in translation efficiency (***9***). Accordingly, different production strategies should be chosen for different plasmid copy numbers in order to obtain a productive process. For applications such as DNA production for gene therapy, high plasmid copy number is an important objective function (***10***).

1.1.2. Chromosomal Integration

Chromosomal integration of the gene of interest is a powerful alternative for overcoming problems of expression stability in plasmid-based systems. In addition, the host does not bear the burden of plasmid maintenance and replication. Chromosome integration is especially suitable for metabolic engineering of the host (***11***) (*see* Chapters 7–10, 20, 22, 24, 26, 29–33). However, several disadvantages over plasmid-based systems exist for recombinant protein production. Adequate integration of a foreign gene in the chromosome is labor-intensive and time-consuming. Moreover, chromosome integration typically results in lower production rates than with plasmid-based systems due to a low copy number of the recombinant gene (***12***). Nonetheless, Olson et al. (***13***) have described methods for obtaining multiple gene integration into the chromosome that yield similar expression levels to those achieved by plasmid systems. The recombinant cells obtained are able to grow in the absence of antibiotics without any reduction of recombinant protein yields. This approach also had the advantage of not infringing patents. Other strategies for achieving chromosome integration in *E. coli* have been discussed by Balbás and Gosset (***11***).

Chromosome integration is the strategy of choice for the commercial expression of recombinant proteins by animal cells. In this case, the long and intricate procedure invested in host development is easily compensated with a stable host. Several strategies to obtain chromosomally integrated genes in animal cells have been developed and are summarized by Twyman and Whitelaw (***14***). Still, a major problem encountered with chromosomal integration is the possibility that the gene of interest will become integrated into an inactive region of chromatin. Among the various strategies used to overcome such a problem (***14,15***) is the use of locus control regions (LCRs), which ensures transcriptional regulation of the transgene (*see* **Subheading 3.3**).

1.1.3. Viral Vectors

An easy and very effective way of delivering the gene of interest is through viral vectors. Viruses have evolved to deliver their genetic material to the host in an efficient and nondestructive way. Some viral vectors, such as retroviruses, promote integration of the viral genome into the cell's chromosome. Many others are used for transient expression. In these cases, recombinant protein production occurs only during certain stages of the life cycle of the virus. Common viral vectors are summarized in **Table 1**, and are described with more detail in Twyman and Whitelaw (***14***). The simplicity of virus-driven protein expression makes it useful for production in higher eukaryotes, as obtaining stable recombinant animal cells may be a tedious and long procedure. Transient

Table 1
Common Viral Expression Vectors for Recombinant Protein Production

Viral vectors	Genetic material	Observations
Adenovirus	Double-stranded linear DNA	Reach high titers (10^{12}–10^{13} pfu/mL); some subgroups are oncogenic; has a wide host range; gene transfer is very efficient; are easy to manipulate in vitro.
Adeno-associated virus	Single-stranded DNA that stably integrates into the host's genome	Naturally defective viruses; can enter in a latent infection that results in long-term transgene expression.
Alphavirus (Semiliki forest virus and Sindbis virus)	Single-stranded positive sense RNA	Host range includes insects and mammals; high recombinant protein concentration; RNA will not integrate into the host's chromosome. They are not pathogenic.
Baculovirus	Double-stranded circular DNA	Hosts are arthropods; may deliver genetic material to mammalian cells (*see* **ref. *16***). Safe, easy to manipulate, and highly productive.
Herpes virus	Double-stranded linear DNA	Broad host range; can infect neurons; can carry up to 50 kbp of foreign DNA.
Poxvirus (vaccinia)	Double-stranded linear DNA	Wide host range; strong expression levels; cytoplamic transcription.
Retrovirus	Single-stranded RNA	Integrates DNA into host's genome; easy to manipulate; some are oncogenic; infection efficiencies close to 100%.

Data from ref. ***14.***

expression is often utilized for rapidly generating sufficient amounts of protein for laboratory scale applications or for preliminary testing of drug candidates. Once a promising molecule is identified, a stable cell line can be generated. Viral expression systems may also find a niche for industrial protein production. For example, the insect cell-baculovirus expression vector system (BEVS) is utilized to commercially produce sev-

eral recombinant proteins (***16***). Moreover, BEVS is especially suitable for the production of vaccines. A relatively new field of application for viral vectors is gene therapy, but this will not be discussed here because requirements and characteristics are different from those for recombinant protein production.

Recombinant gene expression from viral vectors comprises specific issues that are different from those of plasmid- or chromosome-based systems. The use of viral vectors involves a process with two different phases: first, cells are grown to a desired cell density, and then they are infected with the virus of interest. In addition, a virus-free product must be guaranteed for most applications; thus, special considerations are required during purification operations. Virus infection can be comparable to induction in other systems. One of the most important limitations of expression systems based on viral vectors is the quality of the viral stock. Serial in vitro passaging of stocks can result in the appearance of mutant viruses known as defective interfering particles (DIP). The genome of DIP has several deletions that make their replication faster than that of intact viruses. Therefore, DIP compete for the cellular machinery and can drastically reduce recombinant protein yields (***17***). As DIP replication requires a helper virus, in this case the complete virus, their accumulation can be avoided by using multiplicities of infection (MOI) lower than 0.1 plaque-forming unit (pfu) per cell. At such low MOI, the probability that both an intact virus and a DIP will infect the same cell is very low (***16***).

Two parameters of particular relevance during expression with viral vectors are the MOI and the time of infection (TOI). Time of infection refers to the cell concentration at which virus is added to the culture. The TOI should be late enough to allow for sufficient accumulation of cells, but should be early enough for nutrients to remain in an abundant concentration to sustain recombinant protein production. The MOI utilized defines the fraction of the population that is infected at the TOI. At MOI higher than 5 pfu/cell, a synchronous infection can be expected. In contrast, only a fraction of the population will be initially infected when employing MOI lower than 5 pfu/cell, whereas the remaining uninfected cells will be infected during a later stage by the viral progeny generated from the primary infection (***16***). If infection is analogous to induction in other systems, then MOI is equivalent to the strength of induction and gene copy number, and TOI corresponds to the time of induction. The MOI and TOI are closely related and should be selected carefully depending on the particular characteristics of the system of interest. When a high TOI is utilized, then a high MOI should also be employed for maximizing protein yield. On the other hand, low MOI and TOI increase the time of exposure of the recombinant protein to the culture environment, which can be deleterious to labile proteins. Further discussion on this topic can be found in **Subheading 2.1**.

In addition to the mode of infection (MOI and TOI), culture conditions can also affect the infection process (***16,18***). A direct relation between the amount of virus attached to cells and recombinant protein concentration has been observed (***19***). Thus, infection strategies should be aimed at increasing virus attachment, which in turn depends on cell concentration, medium composition, temperature, viscosity, and amount of cell surface available for infection (***18,19***).

1.2. Posttranslational Processing

1.2.1. Folding, Aggregation, and Solubility

Protein folding is a complex process in which two kinds of molecules play an important role: foldases, which accelerate protein folding; and chaperones, which prevent the formation of non-native insoluble folding intermediates (***20***). On occasions, folding does not proceed adequately. This results in misfolded proteins that accumulate in intracellular aggregates known as inclusion bodies. One of the main causes of incorrect protein folding is cell stress, which may be caused by heat shock, nutrient depletion, or other stimuli (***21,22***). Cells respond to stress by increasing the expression of various chaperones, some of them of the *hsp70* and *hsp100* families (***22***). Of particular importance to eukaryotic cells is the "unfolded protein response" that activates transcription of genes encoding chaperones and foldases when unfolded proteins accumulate in the endoplasmic reticulum (***23***). Production of inactive proteins represents an energetic drain and metabolic load, while accumulation of inclusion bodies can cause structural strains to the cell. Accordingly, incorrect protein folding has adverse consequences. For instance, several human pathologies, such as Alzheimer's disease, Parkinson's disease, and Huntington's disease, are characterized by intracellular protein aggregation and accumulation (***21***).

The overexpression of heterologous proteins often results in the formation of inclusion bodies. This phenomenon is still not fully understood, but several explanations have been proposed. For instance, as reviewed by Carrió and Villaverde (***24***), heterologous proteins often reach nonphysiological concentrations, which may promote aggregation. Aggregation can also result from the lack of disulfide bond formation due to the reducing environment of the bacterial cytosol (***5***). Additionally, overexpression of heterologous genes is stressful *per se* and may cause the saturation of the cellular folding machinery (***22***). During heterologous protein production, high rates of expression are required. Proteins may also be larger than those typical of the host, as is the case of mammalian proteins expressed in bacteria. Rapid intracellular protein accumulation (***8***) and expression of large proteins (***22***) increase the probability of aggregation. Accordingly, inclusion body formation is likely to occur during production of recombinant proteins.

Protein aggregation has been observed in bacteria, yeast, insect, and mammalian cells (***20,21,24***) (*see* Chapter 3). Aggregation protects proteins from proteolysis and can facilitate protein recovery by simply breaking the cells and centrifuging the inclusion bodies (***8***). In addition, when the expressed protein is toxic to the host, its deleterious effect can be prevented by producing the heterologous product as inclusion bodies (***25***). In many cases, as with those of the first recombinant proteins that reached the market (insulin and growth hormone), recovery and renaturation operations can be performed in an economically feasible manner (***8***). Accordingly, inclusion body formation not only is desirable, but also can be promoted through molecular biology and/or operation strategies, such as the use of protease-deficient strains, culturing at high temperatures, or designing suitable fusion peptides and amino acid sequences through protein engineering approaches (***24***). If production in inclusion bodies is preferred, solubilization

and renaturation can be performed in different ways (***25***). However, the refolding step is an empirical process that on occasions is very inefficient, with yields usually lower than 10% (***24,26***). Thus, in many cases it may be difficult and expensive to obtain a soluble functional protein after downstream operations. For instance, Datar et al. (***26***) have shown that the overall costs for producing tissue plasminogen activator by an *E. coli* process are higher than those for a mammalian cell-based bioprocess. This is because of the higher expenses incurred during the solubilization and renaturation steps required in the *E. coli* process.

It is impossible to predict whether a protein will aggregate or not in a particular expression system, or how easily it will be solubilized and renaturated (***8,25***). Thus, a soluble protein is generally preferred. Several strategies have been proposed for reducing protein aggregation. Various chaperones and foldases have been stably cloned into hosts to facilitate protein folding (**Table 2; *8,20,22***). However, this strategy is not always successful. It is not possible to predict which chaperone will facilitate folding of a particular protein, or whether more than one chaperone or cofactor will be required. Overexpression of more than one chaperone has been explored with satisfactory results (***22,24***). Protein engineering can also reduce aggregation (***20,24***); changing the extent of hydrophobic regions or using fusion proteins are two successful strategies. Fused proteins often contain a peptide native to the host used. For example, fusing single chain antibodies to an *E. coli* maltose-binding protein allows the production of soluble functional protein in *E. coli* cytoplasm (***27***). Interestingly, it has been observed that proteins accumulated as inclusion bodies can naturally solubilize when heterologous protein production ceases (***24***). Finally, certain additives may facilitate protein folding both in vivo and in vitro. These have been summarized by Fahnert in Chapter 3 of this book.

1.2.2. Proteolytic Processing

Signal peptides, needed to direct proteins to the various cellular compartments, must be cleaved to obtain a functional protein. Upon membrane translocation, the signal peptide is removed by a signal peptidase complex that is membrane-bound to the endoplasmic reticulum in eukaryotes or to the cellular membrane in prokaryotes (***28***). Inefficient removal of the signal peptide may result in protein aggregation and retention within incorrect compartments, such as the endoplasmic reticulum (***29***). Consequently, the yields of secreted proteins can be drastically reduced. To solve this problem, the *E. coli* signal peptidase I and the *Bacillus subtilis* signal peptidase have been overexpressed in *E. coli* and insect cells, respectively (***29,30***). Signal peptidase overexpression increased the release of mature beta-lactamase (***30***) and the processing of antibody single-chain fragments (***29***). Such results demonstrate that low signal peptidase activity can limit the production of recombinant proteins. Despite these promising results, signal peptidase overexpression has rarely been used.

Other proteins, such as proteases, insulin, or penicillin acylase, must be expressed as proproteins because prodomains act as folding catalysts (***31***). In these cases, cells utilize endoproteases to produce the mature active protein (***32***). Accordingly, low endoprotease activity may limit the concentration of a correctly folded mature protein.

Table 2
Some Chaperones or Foldases Utilized to Facilitate Protein Folding

Chaperone/foldase	Host	References
Human hsp70	Insect cells	***20***
BiP	Insect cells	***20***
Calnexin and calreticulin	Insect cells	***20***
Bacterial protein disulfide isomerase (PDI)	Insect cells, *E. coli*	***8,20***
Peptidylprolyl *cis-trans* isomerase	Insect cells	***20***
Trigger factor (TF)	*E. coli*	***22***
DnaK	*E. coli*	***22***
GroEL/ES	*E. coli*	***22,24***
ClpB	*E. coli*	***22***
Skp	*E. coli*	***5***
DegP	*E. coli*	***101***
ClpG	*E. coli*	***101***
HtbG	*E. coli*	***101***
Human PDI	CHO cells	***157***
Polyubiquiton	*Kluyveromyces lactis*	***158***
Kluyveromyces lactis PDI	*Kluyveromyces lactis*	***158***

Overexpression of the mammalian endoprotease furin in mouse mammary gland and insect cells increased the concentration of correctly folded product up to eightfold (***33,34***). Similarly, overexpressing yeast's Kex2p increased processing of proopiomelanocortin by baby hamster kidney (BHK) cells (***32***).

Another type of proteolytic processing is the removal of the N-terminal methionine. This processing is performed by a methionine aminopeptidase (MAP) and occurs only in proteins in which the second amino acid is alanine, glycine, proline, serine, threonine, or valine (***35***). Removal of N-terminal methionine is a common problem during expression by *E. coli*. Overexpression of recombinant proteins may saturate MAP or deplete required metal cofactors (***35***). Similarly to other enzymes, MAP has been overexpressed in *E. coli* to solve such a problem. Using this strategy, Hwang et al. (***36***) were able to increase *N*-methionine removal by 40%, but recombinant gluthatione S-transferase concentration was reduced 10%. Since Vassileva-Atanassova et al. (***37***) did not find a correlation between the extent of *N* -methionine removal and recombinant protein concentration in two strains with different intrinsic *N*-methionine removal ability, the reduced yield observed by Hwang et al. (***36***) could be attributed to the higher metabolic load that results from overexpression of two recombinant genes. Another alternative for *N*-methionine removal is the construction of fusion proteins, where the -*N*-methionine is removed along with the fusion peptide either intracellularly or during a later in vitro enzymatic removal stage (***35***).

1.2.3. Glycosylation

Glycosylation is a very complex posttranslational modification that requires several consecutive steps and involves tens of enzymes and substrates (**Figs. 1, 2**). It usually occurs in the endoplasmic reticulum and Golgi apparatus of eukaryotic cells, although *N*-glycosylation has been detected in proteins produced by bacteria (***38***). Three types of glycosylation exist: *N*-(glycans linked to an Asn of an AsnXaaSer/Thr consensus sequence, where Xaa is any amino acid), *O*-(glycans linked to a Ser or Thr), and C (attached to a tryptophan) linked. Of these, C-linked glycosylation has hardly been studied and little is known about its biological significance (***39***). *N*-linked glycosylation is the most studied and is considered as the most relevant for recombinant protein production. In many cases, glycosylation determines protein stability, solubility, antigenicity, folding, localization, biological activity, and circulation half-life. Glycosylation profiles are protein-, tissue-, and animal-specific (***40***). Nonauthentic glycosylation may trigger immune responses when present in proteins for human or animal use (***40***). Therefore, authentic glycosylation is especially relevant for recombinant proteins to be utilized as drugs.

The *N*-glycosylation pathway is depicted in **Figs. 1** and **2**. Several bottlenecks can be expected from the complexity of the process. Moreover, different glycosylation sites are often glycosylated in different ways (***41***). Recombinant proteins may present macroheterogeneous (differences in site occupancy) or microheterogeneous (differences in the structures of oligosaccharides between glycosylation sites) glycosylation (***42***). First, the synthesis of the dolicholphosphate oligosaccharide can limit the extent of glycosylation. This can occur from a reduction of the lipid pool. In addition, the concentration of lipid-linked oligosaccharides has been reported to be cell-cycle-dependent (***43,44***). As an attempt to solve this, dolicholphosphate has been fed to Chinese hamster ovary (CHO) cells, producing recombinant proteins (***45,46***). Although dolicholphosphate was internalized (***46***), no increase in site occupancy was observed upon its addition (***45,46***). On the other hand, a reduced pool of sugar nucleotides, the activated sugar donors required for oligosaccharide synthesis, limits the buildup of the G3M9N2Dol PP precursor (where G is glucose, M is mannose, and N is *N*-acetylglucosamine) and reduces the glycosylation site occupancy (***47,48***). Limitation of sugar nucleotide donors occurs upon prolonged glucose or glutamine starvation (***47,48***). The availability of sugar nucleotide donors also affects microheterogeneity, as each step of the building of oligosaccharide chains in the Golgi apparatus requires nucleotide sugars (**Fig. 2**). To alleviate such a problem, sugar nucleotide precursors have been added to the culture medium. With this approach, sialylation by Chinese hamster ovary (CHO) and genetically engineered insect cells has been increased through feeding of *N*-acetylmannosamine (***49,50***). Another factor that can affect glycosylation is the transport of sugar nucleotides to the endoplasmic reticulum or Golgi apparatus. Gu and Wang (***49***) and Hills et al. (***51***) proposed this when an increase in nucleotide sugar pool did not result in a proportional increase of the extent of protein glycosylation.

Another possible factor affecting glycosylation is the presence of glycosidases, either intracellularly or in the culture medium. This can be a major problem when

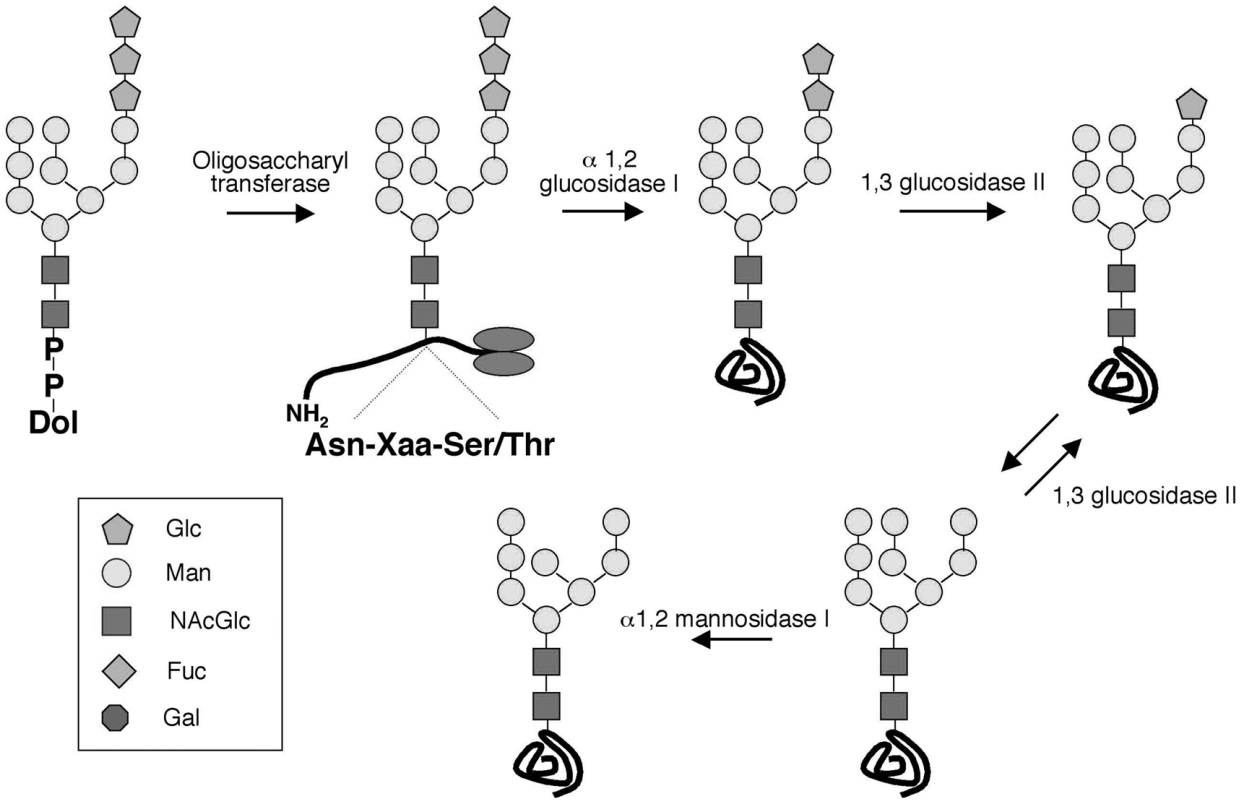


Fig. 1. *N*-glycosylation in the endoplasmic reticulum. First, a lipid-linked oligosaccharide is synthesized in the endoplasmic reticulum (depicted as the Dol-P-P-oligosaccharide). Glycans are then transferred to the nascent peptides. Thereafter, glycan processing proceeds as depicted. Dol = dolichol; Glc = glucose; Man = mannose; NAcGlc = *N*-acetylglucosamine; Fuc = fucose; Gal = galactose.

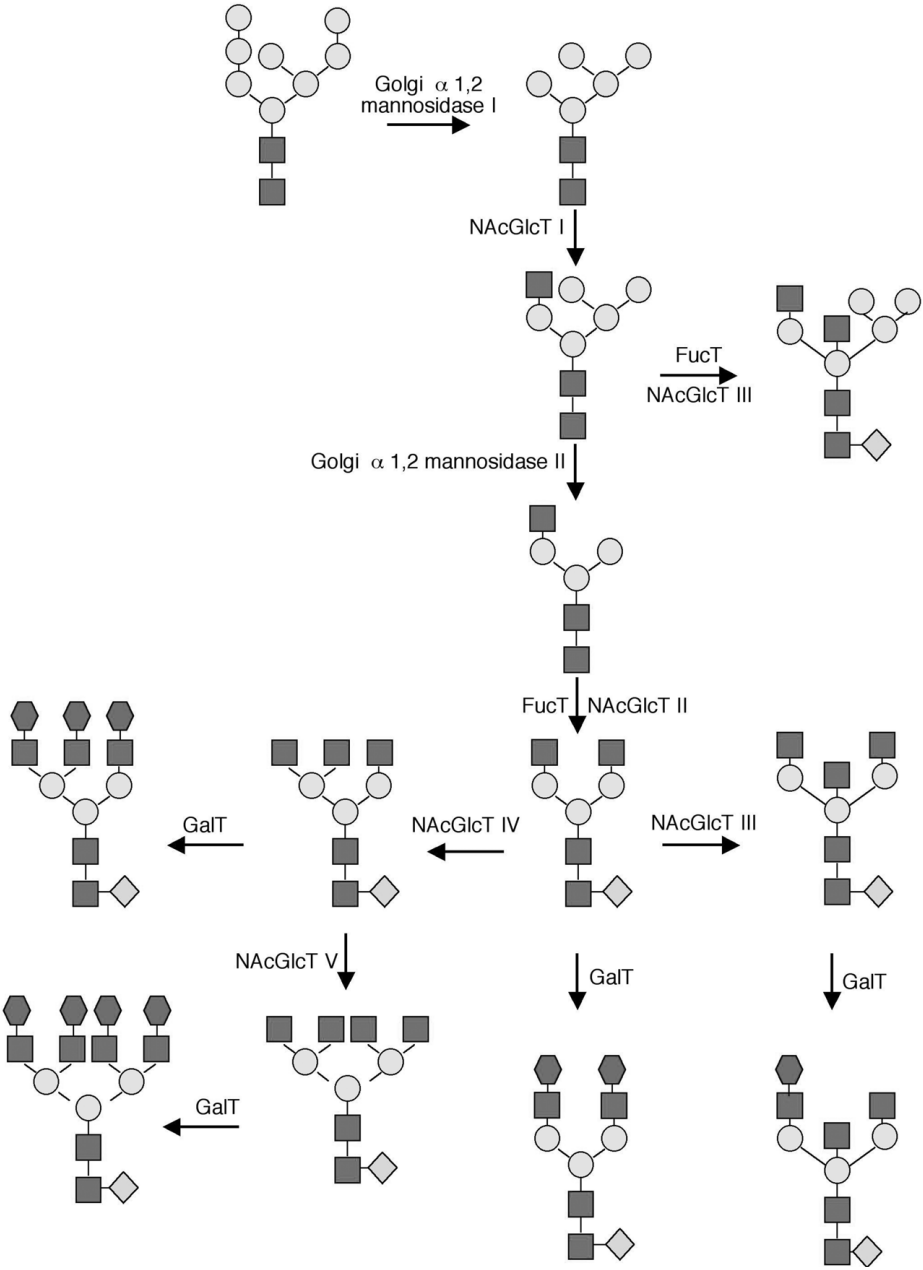


Fig. 2. *N*-glycan processing in the Golgi. The protein is not depicted for clarity. Pathways shown are only typical processing routes, others may occur. T refers to transferase. Symbols are the same as those in **Fig. 1**. FucT may act in the different sites shown. Galactosylated glycans are substrates for sialyltransferase, and thus can be sialylated (pathway not shown).

expressing proteins in insect cells, as an intracellular hexosaminidase activity results in the accumulation of paucimannosidic glycans (containing three or fewer mannose residues and the NacGlc core) (***52***). Moreover, Gramer and Goochee (***53***) have detected and characterized the activities of sialidase, β-galactosidase, α-hexosaminidase, and fucosidase in supernatants of CHO cell cultures. Sialidase activity increased upon cell lysis due to the release of cytoplasmic enzymes (***54***). High extracellular sialidase activity resulted in decreased sialylation of glycans attached to recombinant human antithrombin III (***54***). This problem can be solved through the addition of glycosidase inhibitors or by harvesting the product before extensive cell lysis occurs (***53,54***).

Culture conditions can also affect glycosylation. For example, pH can affect the activity of extracellular glycosidases. The concentration of toxic byproducts, such as ammonia, CO_2, and hyperosmotic conditions, can reduce sialylation and the extent of *N*- and *O*-glycosylation (***55,56***). Cell growth rate and protein production rate also influence glycosylation. For instance, Andersen et al. (***45***) observed a direct relation between site occupancy and the fraction of cells in the G0/G1 phases of the cell cycle. Moreover, decreasing temperature of CHO cell cultures significantly increased the degree of sialylation of secreted alkaline phosphatase (***57***). Reduced growth rate may result in a reduced protein production rate, which in turn increases the extent of glycosylation (***42***). Protein glycosylation is a dynamic phenomenon that changes as culture time progresses. Andersen et al. (***45***) and Yuk and Wang (***46***) have found that glycosylation levels increase with increasing culture time until the onset of cell death. As with *N*-glycosylation, nonauthentic *O*-glycosylation profiles can also elicit an immune response toward the recombinant product (***58***). Strategies proposed for *N*-glycosylation can also improve the amount of sialylated and galactosylated *O*-glycans.

1.2.4. Other Posttranslational Modifications

Other posttranslational modifications, such as myristoylation, palmitoylation, isoprenylation, phosphorylation, sulfation, C-terminal amidation, β-hydroxylation, and methylation, are less common than glycosylation, but may be important for certain recombinant proteins. In general, the extent of modification depends on the host utilized, being the modifications performed by higher eukaryotic cells closer to those found in human proteins (*see* **Subheading 3**).

1.3. Transport and Localization

As already discussed, recombinant proteins may be directed to different cellular compartments by signal peptides or through fusion proteins. Different sites of protein localization have different advantages and disadvantages, which are summarized in **Table 3**. Intracellular accumulation often results in high protein amounts and allows an easy recovery of concentrated protein along with cells (***35***). Nonetheless, purification of the product from the protein-rich cell extract may be difficult. In contrast, the product of interest usually constitutes the major component when it is secreted to a low-protein or protein-free medium. This can greatly facilitate its purification. Nonetheless, secreted proteins will be highly diluted and bottlenecks in the secretion pathway can further

Table 3
Possible Locations and Conditions of Recombinant Protein Accumulation

Protein design	Location	Soluble	Advantages	Disadvantages
Native sequence	Cytoplasm	yes	Direct purification with high yield recovery. High level of expression.	Susceptible to proteolysis. High cellular native protein content.
	Cytoplasm	no	High-level expression. May prevent proteolysis. Toxicity effects of protein to cell may be avoided. Easy partial purification.	Protein folding must be carried out. Recovery of purified native protein can be low or even zero.
Fusion protein	Cytoplasm	yes	High-level expression. Purification may be aided with affinity-tagged protein. Solubility and stability may be enhanced by fusion partners.	Site-specific cleavage of fusion peptide required. Overall yield may be low.
Fusion protein directed to secretion	Cytoplasm	no		Signal peptide unprocessed, purification usually not attempted.
	Periplasmic space[a]/medium	yes	Ease of purification	Expression level and recovery may be low. Diluted product.

Adapted from Wingfield (***35***).
[a]In Gram-negative bacteria.

reduce their accumulation in the culture medium (***20***). Accordingly, concentration operations, such as ultrafiltration, are always used prior to other purification stages when dealing with secreted proteins.

Protein localization is especially relevant when expressing recombinant proteins in *E. coli*. Accumulation in the periplasm often results in soluble and correctly folded proteins, whereas cytoplasmic localization yields an inactive and insoluble product (***35***).

The characteristics of the protein should be considered when deciding the site of accumulation. Small proteins susceptible to proteolysis should be produced in *E. coli* as inclusion bodies. Apart from intra- or extracellular accumulation, certain applications may require recombinant proteins to be targeted to the cell membrane, usually through fusion proteins. This is the case of virus or phage display, where protein localization in the virus surface allows for rapid screening and isolation of the desired phenotype, which is coupled to the corresponding genotype (***59***). The transport efficiency of the protein of interest depends on the signal peptide utilized, which should be chosen according to the host. Nonoptimal selection of the signal peptide results in intracellular protein accumulation and aggregation (***20***).

2. Bioengineering Approaches to Solve Common Problems Associated With Heterologous Gene Expression

Bioprocess engineering plays a crucial role when the goal of recombinant protein production is to obtain as great amounts as possible of a high-quality product. As already discussed, bioprocess conditions affect not only the amount of protein obtained, but also its solubility and its posttranslational modifications. The biology of the host, and the molecular biology tools utilized for its modification, should be taken into account when defining bioprocess conditions. For example, different approaches are required when employing either high- or low-copy-number plasmids. Similarly, animal cells have very different requirements from bacteria or fungi. Experience has shown that the best results are obtained when both molecular biology and bioengineering approaches are used. Some common strategies for improving recombinant protein production through manipulation of the culture conditions will be discussed in the following sections.

2.1. Induction Strategies

Recombinant genes can be placed under a variety of promoters. The promoter selected will determine whether gene expression is constitutive or inducible (***60***). Constitutive gene expression may increase plasmid instability because the metabolic load of recombinant protein production is constantly present (*see* **Subheading 1.1.1**). Thus, constitutive promoters are normally chosen when recombinant gene expression does not significantly affect the growth rate of the host. In many situations the best conditions for cell growth are different from those for recombinant protein production (***15***). In such cases, inducible systems are preferred—i.e., systems in which induction is performed after a particular cell density has been obtained. Different types of stimuli can be utilized for induction (***12***). Induction may depend on starvation of a nutrient and/or the addition of an alternative nutrient that turns on specific molecular machinery, such as the *lac* operon. Other inducers include osmolarity, pH, or temperature shifts, anaerobiosis, antibiotic addition, and the like. Several considerations should be made when choosing an inductive system. Induction should be simple, economical, and efficient. In addition, the inducer should not have negative effects on cell viability and recombinant product quality, and should not complicate downstream operations. Finally, the chosen system should be efficiently repressed in the absence of the inducer (***12***). An advantage

of physical or physicochemical induction, such as temperature, pH, dissolved oxygen tension, and osmolarity shifts, is that chemicals, which may be undesirable in the final product, are not added. Moreover, these induction methods are easy to implement and are inexpensive at laboratory and pilot-plant scales.

Industrial recombinant protein production requires additional considerations of the type of inducer employed. Among these are the deficient mass, heat, and momentum transfers often observed in large-scale bioreactors (***61***). For instance, mixing times (time required to achieve homogeneity) in large-scale animal or plant cell culture vessels (10,000 L) can be in the order of 10^3s. Thus, up to 16 min would be required for the inducer to be homogeneously distributed in the reactor under this extreme situation. This can be solved by using several feeding ports, if the inducer is a chemical added to the vessel (***62***). However, such an approach cannot be utilized for other type of induction, such as temperature changes. Reducing or increasing the temperature of a large-scale vessel may be very expensive and ineffective. Moreover, the rate of temperature change can affect recombinant protein yield (***63***). In conclusion, the dynamics of the production process should be considered.

Once a system of induction has been chosen, induction strategies must be planned. The first consideration should be the effect of recombinant gene expression on cell growth and physiology. In some cases, usually when low plasmid copy numbers are present, recombinant protein production does not affect the specific growth rate (e.g., ***64,65***). Thus, higher recombinant protein yields are obtained by inducing foreign gene expression as early as possible, even at the time of inoculation (***64,65***). When cell growth is significantly inhibited by the expression of a recombinant gene, sufficient buildup of biomass should be allowed before induction (***66***). However, extreme cell concentrations may reduce production of the recombinant protein, as nutrient limitation may occur. The importance of adequate nutrient feeding after induction was investigated by Yazdani and Mukherjee (***66***). They observed a 10-fold increase in recombinant streptokinase concentration when concentrated medium was fed after induction (*see* **Subheadings 2.2** and **2.3**). A similar effect has been observed when expressing recombinant proteins through the BEVS. In this case, very little recombinant protein is produced if infection with the recombinant baculovirus is performed above an optimum cell concentration. This phenomenon has been called the "cell-density effect" and can be overcome through adequate nutrient feeding strategies (***16***). It should be noted, however, that infection at extremely high cell concentrations (in insect cells, above 14×10^6 cells/mL) may drastically reduce recombinant protein yields even when nutrients are available (***67***). The reasons for this are still unknown, but it is possible that a trace element that is not fed limits yields.

The mode of induction can also affect the solubility of the recombinant product (***68***). Cells must be actively growing at the time of induction to reduce protein aggregation. Accordingly, Eriksen et al. (***65***) observed an increase in solubility of a recombinant protein when expression was induced in the very early exponential phase, in comparison with induction at later times or in the lag phase. As discussed previously, a common strategy to increase protein solubility is by reducing the culture temperature after induction. This gives an advantage to cold-shock over heat-shock promoters when aggrega-

tion must be avoided. However, maintaining the culture at low temperatures drastically decreases growth rate. Therefore, cell concentration should be as high as possible at the time of induction when cold-shock promoters are used (***68***).

The magnitude and length of induction also affect recombinant protein yields. Low inducer concentration may result in an inefficient induction (low recombinant protein yields), whereas expensive inducers added in excess can result in an important economic loss or in toxic effects, including reduced cell growth and/or recombinant protein concentration. A saturation-type relationship between inducer concentration and maximum recombinant protein volumetric or specific yields has been reported for the *lacZ* promoter induced with IPTG (***64***). Thus, inducer concentration should be maintained at or slightly higher than the critical concentration (the concentration below which recombinant protein yield becomes a function of inducer concentration). As observed by Ramírez et al. (***64***), IPTG concentration between 0 and 1 m*M* did not affect *E. coli* specific growth rate or maximum cell concentration. However, such a behavior must be characterized for the particular host/vector/protein employed. In the case of temperature-induced promoters, the temperature of induction and the duration of the temperature shift have an important effect. For example, Gupta et al. (***69***) expressed *lacZ* under the T7 system using the $\lambda P_L >$ heat-shock promoter. Several choices exist for inducing such a system. Namely, temperature can be increased to 42°C for a given period of time, or maintained at 42°C until the end of the culture (***69***). As cell growth ceases at 42°C, a high cell concentration must be present before induction for the culture to be productive when the temperature is maintained at 42°C. In contrast, Gupta et al. (***69***) found that a heat shock of 2 min did not arrest cell growth and was optimal for recombinant protein production. Therefore, maximum recombinant protein yields were obtained when induction was performed in the early growth phase.

2.2. Growth Control

Growth rate affects several parameters that determine recombinant protein accumulation rate. Among them are the percentage of substrate utilized for cellular maintenance, RNA polymerase activity, ribosome number, plasmid stability, plasmid copy number, plasmid multimerization, and the distribution of cells in the cell-cycle phases (***70–73***). Thus, it is possible to control recombinant protein production through growth rate. Growth rate can be manipulated through nutrient availability. Namely, the main carbon or nitrogen source can be maintained at a predetermined concentration to obtain the desired growth rate. Such a manipulation can be achieved through fed-batch or continuous cultures (***74,75***). Dissolved oxygen, an essential nutrient for aerobic cells, can also be utilized to control growth rate. Temperature also affects growth rate by changing the rate of the reactions occurring in the culture vessel. Temperature is an especially effective tool for arresting growth in animal cells, as at low temperatures cells remain viable, mostly in the G1 phase of the cell cycle (***57***). It should be noted that all these factors can have additional particular effects besides modifying growth rate. For instance, reducing the growth rate by limiting nutrient concentration may reduce the production of undesirable metabolites by increasing the metabolic efficiency (***8,76,77***). Molecular biology approaches can also be utilized to manipulate growth rate. For example,

Kaufmann et al. (***57***) introduced the cell-cycle-arresting gene p27 under the control of a tetracycline-repressible promoter in CHO cells. In this manner, they divided the process in two: first, a stage of active cell growth, and second, after tetracycline decomposition, a stage in which recombinant protein is produced and cell growth is arrested in the G1/S restriction point. As a result, the concentration of recombinant-secreted alkaline phosphatase (SeAP) increased 17 times.

Contradictory information can be found in the literature, where protein production rate has been reported to increase (***72***) or decrease with specific growth rate (***73***), or show no relation at all (***7***). Therefore, the effect of growth rate on protein productivity cannot be generalized. The first consideration to be made is whether protein accumulation is associated with cell growth. This is usually the case, except when the recombinant protein is toxic to the cell, when it severely reduces growth, or when growth drastically decreases plasmid stability. When recombinant protein production is growth-associated, sustained growth should result in higher protein concentrations and induction can be performed in the beginning of the culture (*see* **Subheading 2.1.**). On the contrary, when protein production is not growth-associated, an optimized process should be divided into a growth phase and a production phase. In the latter phase, cell survival and plasmid maintenance should be promoted instead of cell growth. The host and the plasmid construction determine the effect of growth rate, as has been shown by Saraswat et al. (***73***). In many cases, the relation between growth rate and the amount of recombinant protein cannot be explained. In many others, changes in recombinant protein yields have been correlated with plasmid copy number, stability, or multimerization. Generally, growth rate is inversely related to plasmid stability (***73,78***), although the contrary has also been reported (***7***), suggesting that each particular case should be evaluated individually. It has been proposed that high growth rates and protein production rates represent a stressful condition that may affect plasmid replication and multimerization (***73***). On the other hand, reduced growth rates increase the plasmid copy number of continuously replicating plasmids (***78***).

In addition, cells in different stages of the cell cycle produce different amounts of recombinant protein or are less susceptible to infection. Thus, suitable growth rate control strategies must be imposed during the protein production phase. For example, Leelavatcharamas et al. (***79***) used control of the cell cycle to improve production of interferon γ, a growth-associated product.

2.3. Bioreactor and Operation Strategies

The main objective of a bioreactor, besides containment, is the control of environmental parameters in predetermined values. The number of parameters that can be manipulated depends on the complexity of the bioreactor. It can range from only temperature, when static culture flasks are introduced in an incubator, to several parameters in a fully instrumented vessel. Among the conditions that can be controlled are dissolved oxygen, pH, temperature, agitation rate, redox potential, dissolved carbon dioxide, cell concentration, cell growth, substrate concentration, inlet gas flow and composition, volume, pressure, fluid dynamics, and power input. Lidén (***80***) proposed to call the set of environmental conditions present in a bioreactor the “envirome.” The

envirome results not only from the action of process parameters manipulated by the operator, but also from the direct interaction of cells with their environment. The envirome interacts with several steps of the recombinant protein production process, namely cell growth, cellular metabolic state, transcription, translation, and posttranslational modification. From the importance of the envirome, it can be seen that bioreactors have an immense potential for increasing recombinant protein productivity.

Of the parameters listed above, dissolved oxygen tension (DOT) has received special attention, because oxygen has a low solubility in water and is difficult to deliver to the culture broth (***81***). The problem aggravates at very high cell concentrations, as higher amounts of oxygen must be transferred to the culture medium to satisfy demand. Cultures need to be fully aerated and homogeneous to avoid alcoholic or acid fermentation in bacteria, yeast, and animal cell cultures (***82,83***). Consequently, bioreactors are designed to increase the oxygen transfer rate (OTR) as much as possible. In the case of bioreactors employing suspended cells, homogeneity is achieved by both the action of the impellers and the liquid motion induced by gas sparging. Nonetheless, bioreactors employing cells immobilized to a variety of supports are needed in some circumstances. This is the case of anchorage-dependent animal cells. In other cases, immobilizing cells that would otherwise grow freely in suspension is needed to attain high cell concentrations and high productivities. In these situations, homogeneity can be achieved by agitation if cells are immobilized in supports that become suspended during operation, such as microcarriers. When a fixed matrix configuration is employed, homogeneity can be achieved by increasing medium flow rate and by suitable bioreactor design. Several strategies for operating bioreactors with immobilized cells are described elsewhere (***61***). Palomares and Ramírez (***61***) have discussed the characteristics and problems of the different types of bioreactors upon process scale-up.

In general, a DOT higher than 20% (with respect to air saturation) does not limit growth, unless transfer from the liquid to the cells is restrained by diffusion through additional resistances, such as when cells form aggregates or pellets or are immobilized. In these cases, a 50% DOT in the bulk liquid may be required to sustain growth of agglomerated cells (***84***). In addition to its effects on cell growth, oxygen privation can drastically increase plasmid instability (***10,85***). For instance, Li et al. (***86***) observed an increase in plasmid content at higher DOT, but no significant effect on recombinant protein yields was detected. Thus, they hypothesized that plasmid replication is suppressed and gene expression increased in anaerobic conditions. It should be noted that the effect of DOT on recombinant protein yield was strain-dependent. Oxygen is also required for maturation of proproteins, as penicillin acylase (***87***). Among the strategies utilized to cope with the problem of poor oxygenation of cultures is the expression of *Vitreoscilla* hemoglobin in the host. This allows efficient growth at limiting dissolved oxygen concentrations and improves recombinant protein yields (***88***). In contrast to oxygen limitation, an oversupply of oxygen can cause oxidative stress to cells or oxidative damage to proteins (***85***). Some proteins, such as cylohexanone monooxygenase, are very susceptible to oxidation and should be produced at DOT of 0% (***89***). Special considerations should be made when utilizing temperature-inducible promoters, as oxygen is less

soluble in water as temperature increases. If a reactor is near its maximum OTR capacity and temperature is increased, the resulting OTR may not be sufficient to sustain recombinant protein production.

Oxygenation of cultures employing fragile cells, such as animal cells and filamentous fungi, is often problematic, as sparging and agitation are limited to shear stresses that are not harmful. Typical energy dissipation rates in bioreactors are usually below those deleterious to animal cells; thus, damage from agitation should not be expected (***90***). However, bubble rupturing in sparged cultures liberates very high amounts of energy that kill almost every cell in the surrounding area (***91***). As the energy liberated from bubble bursting is inversely related to bubble size, large bubbles should be utilized in fragile cultures (***92***). The area for oxygen transfer decreases as bubble size increases. Hydrodynamic stress can be lethal to cells, or may only infringe sublethal damages that may trigger apoptosis, arrest the cell cycle, increase nutrient consumption rates, change intracellular pH, and reduce recombinant protein yields (***16***). A strategy for reducing shear damage to cells is the use of shear-protective additives, such as Pluronic F68® (BASF), which yields stronger cells by decreasing their membrane fluidity and reduces their attachment to bubbles (***90,93,95***). The situation in cultures of filamentous fungi is different. The morphology of fungi, either dispersed or in pellets, depends on culture conditions (***90***). As agitation speed increases, fungi acquire the form of pellets. Moreover, pellet size decreases as agitation increases. Such changes in morphology are often accompanied by changes in product production, which often decreases as the power applied to the bioreactor increases (***90,96***).

The bioreactor operation mode is another approach to control the environment. Fed-batch cultures are utilized for increasing cell concentration and obtaining high product titers (*see* **Subheading 2.2.**). The control of nutrient concentration can increase metabolic efficiency. For example, maintenance of low glucose concentration can be used to avoid the Crabtree effect (alcoholic or acid fermentation in aerobic conditions due to high concentrations of glucose). The Crabtree effect results in a waste of glucose and the generation of toxic byproducts that often limit recombinant protein yields (***77,97***). On the other hand, nutrient-deprived cultures are more drastically affected by the metabolic burden of foreign gene expression. Glucose, magnesium, phosphate, or oxygen limitation decrease plasmid stability (***9,85***). Meanwhile, the carbon-to-nitrogen ratio also affects plasmid loss and the burden that plasmids impose on cells (***98***). In animal cell cultures, nutrient privation may trigger apoptosis (***99***).

Bioreactor operation mode also influences plasmid stability. High-density cultures and continuous operation are prone to plasmid segregation due to the high number of generations in the culture (***5***). Similarly, large-scale operation increases plasmid instability for the same reason (***81***). Cell immobilization has been observed to reduce plasmid instability (***9***). As mentioned before, the design of two-stage processes, in which cells grow on one stage and are induced and produce recombinant protein in the other, is an interesting alternative to reduce plasmid instability. This can be performed in two-stage systems consisting of chemostats in series. In this arrangement, cell growth is optimized in the first chemostat, and recombinant protein concentration in the second. For example, Sayadi et al. (***9***) utilized such a system to produce cathecol 2,3-dioxygenase in *E. coli*, where plasmid stability was guaranteed by immobilizing the

cells. Two-stage arrangements may be especially useful in systems such as the BEVS and expression in *Bacillus subtilis*, where protein production starts after an infection phase or close to the sporulation phase, respectively.

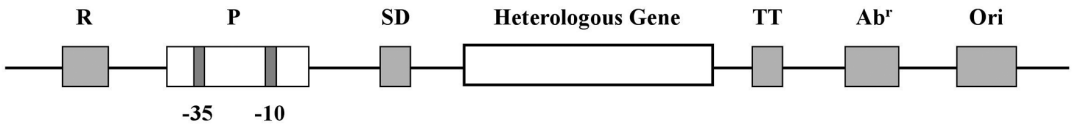
3. Specific Problems and Their Solutions in Different Expression Systems

Recombinant protein production requires integrated bioprocesses that include considerations spanning from molecular biology to downstream processing. Under this notion, the host undoubtedly has a prominent role. Many characteristics of the product are endowed by the host and are influenced by protein concentration and site of accumulation. In general, protein concentration is inversely related to the extent of protein posttranslational processing, and a compromise between quality and productivity must be made. Moreover, the host dictates the molecular biology techniques to be used, production mode, and product recovery strategies. As a rule of thumb, the most simple host expression system that delivers the required quality should be chosen for recombinant protein production. Animal cells, fungi, yeast, and bacteria are commonly used nowadays for the expression of recombinant products.

3.1. Prokaryotes

The Gram-negative bacterium *E. coli* was the first organism utilized for the production of recombinant human proteins. It is still extensively used for industrial applications, as evidenced by a market, only for recombinant pharmaceutical proteins, of $2.9 billion in 1999 (***100***). A large amount of knowledge has been generated about its molecular biology, biochemistry, and physiology (***5,8,101***). *E. coli* is easy to grow to high cell densities (over 100 g/L), and has simple nutritional requirements that can be satisfied with fully defined simple media (***60***). Despite its proven success, recombinant protein production in *E. coli* has several drawbacks that have been addressed through different approaches (***5,8,102,103***). *E. coli* is usually not capable of efficiently producing very long or short proteins, although the successful expression of a 210 kDa protein has been achieved (***104***). Proteolytic cleavage and disulfide bond formation seldom occur, and posttranslational modifications, including glycosylation, acylation, and isoprenylation, are not performed. In many cases, neither of these modifications is required for obtaining an adequate product, and bacteria are the host of choice. In addition, bacteria possess pyrogens and endotoxins that must be totally eliminated from proteins to be injected in animals or humans. Other concerns about the expression of recombinant proteins in *E. coli* include variability in the level of expression, protein solubility, and protein purification. Most of these inconveniences have been approached through genetic manipulations (***103,105***). **Figure 3** summarizes some strategies used for enhancing recombinant protein expression in *E. coli*. (*see* Chapters 5–12 for new applications).

The problem of expression levels traditionally has been solved by using strong promoters and/or intervening on the pathways that include possible rate-limiting steps—namely, novel "metabolic optimization" strategies can be used to finely tune the expression of genes in particular pathways that modulate the final product yield, as well as the expression of the gene of interest (***106***). Fine-tuning can be accomplished by utilizing artificial promoters with different strengths. Such artificial promoters have been constructed by



EXPECTED IMPROVEMENT	MODIFICATION
Enhancement of expression	•Changing promoter •Modification of spacer sequence •Stabilization of mRNA
Solubility	•Fusion of the HG with chaperones •Fusion of the HG with soluble proteins
Transport/secretion protein of interest	•Use of signal peptides •Fusion of the HG with pore forming proteins. •Coexpression of the protein of interest with easily secreted proteins.
Purification	•Fusion of the HG with an easy to purify protein •Tagging the HG with poly-histidine tails, or with a CBD.

Fig. 3. On top is shown the classic organization of a prokaryotic expression vector where (R) is the regulator that exerts its effect on the (P) promoter, whose −35 and −10 sequences are separated by spacer sequences. The Shine Dalgarno (SD) sequence precedes the heterologous gene (HG). Transcription termination (TT), antibiotic resistance marker (Ab^r) and origin (Ori) of replication of the plasmid are necessary for mRNA stabilization, adequate selection and vector copy number, respectively. The table below the diagram indicates some of the modifications of the plasmid that can lead to specific improvements.

modifying the spacing sequences between the –10 and –35 regions of constitutive promoters from *Lactococcus lactis*, which are also useful in *E. coli* (***107***). In this way, libraries of artificial promoters with different strengths can be generated for each host. Such a technology has been patented (***108***). Often, metabolic optimization requires the simultaneous regulation of expression of various genes. Different promoters can be utilized for each gene, or various genes can be placed under the same promoter in an operon. In the latter case, expression can be regulated by increasing the stability of each coding region through the introduction of stabilizing sequences, such as those forming hairpins (***109***).

Plasmid copy number is directly related to recombinant protein productivity (*see* **Subheading 1.1.1.**), and is regulated by plasmid replication. ColE1-type plasmids are found in Gram-negative bacteria and are part of most cloning vehicles used today. Their replication requires an RNA preprimer called RNA II (***110***). RNA II must be cleaved by the host's RNAase H to release the 3' OH that is used by the DNA polymerase I to initiate replication. Control of the initiation of ColE1 replication is mediated by the interaction of RNA II with an antisense RNA, RNA I, that impedes cleavage of RNA II (***111***). However, control of plasmid replication can be lost when a recombinant protein is overproduced. This is due to an increased pool of uncharged tRNAs provoked by high amino acid consumption rates. Such uncharged tRNAs bind to RNA I, disturbing the natural plasmid replication control mechanism. To avoid this, Grabherr et al. (***112***) modified the nucleotide sequence of RNA I, preventing the binding of tRNA. Such a strategy allowed a better control of the recombinant protein production process, and reduced the metabolic burden that occurs upon uncontrolled plasmid replication. Plasmid copy number can be modified by mutating RNA I or RNA II, or by altering their expression rates. Further control of plasmid replication can be obtained by altering the structure of RNA I and RNA II (***112***) (*see* Chapter 4).

Apart from the strategies discussed in **Subheading 1.2.1.**, many molecular biology approaches have been employed to deal with the problem of recombinant protein accumulation in inclusion bodies when using *E. coli* (***24***). Non-membrane-bound proteins that are correctly folded should be reasonably soluble in aqueous solution, and it is believed that the amino acid sequence at the amino and carboxy termini play a role in their solubility (***113***). Likewise, recognition by proteases is dependent on the polarity of the residues at these termini. Accordingly, Sati et al. (***113***) analyzed the overexpression of a cytoplasmic protein from *Plasmodium falciparium* in *E. coli*. Various constructs bearing extra amino acids at the N- and C-termini were designed. Results indicated that the presence of polar amino acids in the C-terminus and the length of the additional sequence enhanced solubility and stability of the recombinant protein. Similar results on the stability of other recombinant proteins by addition of C-terminal tails have been summarized by Sati et al. (***113***; **Table 3**). As already discussed, fusion proteins are a strategy commonly used to increase recombinant protein solubility. Davis et al. (***114***) proposed a rational strategy for the identification of possible fusion partners that could confer solubility to proteins expressed in *E. coli*. Possible fusion partners were identified from a statistical solubility model. Proteins predicted to be highly soluble were useful to increase the solubility of recombinant proteins when fused to them.

The disulfide bond (Dsb) protein A and DsbB, from the oxidizing pathway, and DsbC and DsbG, from the isomerizing pathway, are found in the periplasmic space of *E. coli* (***115***). These enzymes catalyze the formation of disulfide bonds in nascent proteins. Moreover, both DsbC and DsbG have been shown to have chaperone activity, promoting reactivation and folding and suppressing aggregation (***116,117***). Maskos et al. (***118***) have recently shown that coexpression of DsbC with a complexly folded protein can improve disulfide bond formation in the periplasm.

Gram-positive bacteria, such as *B. subtilis*, have also been utilized for recombinant protein production, with the advantage that they can secrete large amounts of properly folded product and contain low concentrations of pyrogens (***119***). However, recombinant plasmids are not stable in *B. subtilis* and chromosomal integration is the only way to obtain a stable recombinant cell. Yields are lower than those of Gram-negative bacteria, due mostly to the high activity of endogenous proteases. In fact, one of these proteases, subtilisin (produced by *B. subtilis)*, is produced in very large amounts to satisfy the detergent industry. Protein engineering has been utilized to produce subtilisin with new properties. *B. subtilis*, in contrast to *E. coli*, is generally recognized as safe (GRAS) and can be used for the production of proteins for the food industry (***120***). However, *B. subtilis* responds to stress by producing proteases and sporulating, consequently reducing recombinant protein concentration *(**121***). Medium composition, specifically the concentration of some salts and peptone, can prevent sporulation and increase the concentration of recombinant protein (***122***). Strains of *B. subtilis* that produce lower concentrations of proteases have been utilized for the production of recombinant proteins. Moreover, asporogenous mutants have been isolated (***123***). The utilization of Gram-positive bacteria may find a niche for the production of recombinant proteins, most probably for the production of proteins for nonpharmaceutical industrial applications. Additionally, their use in the synthesis of correctly posttranslationally modified nonribosomal peptide synthetases proves to be appealing, as Doekel et al. (***104***) stably expressed these complex enzymes in *B. subtilis*.

3.2. Yeast and Fungi

Yeasts have been utilized by humans since the Neolithic age (***124***). Their various applications in the food industry and for single-cell protein production has taken yeast fermentations to the largest volumes ever performed (***81***) (*see* Chapters 16–22). The yeast *Saccharomyces cerevisiae* was the first yeast species to be manipulated for recombinant protein expression (***125***), and many proteins have been produced in it. Due to its many applications, excellent knowledge of *S. cerevisiae* molecular biology and physiology has accumulated (***125***). *S. cerevisiae* is GRAS and, like other yeasts, can secrete recombinant proteins to the culture medium. Moreover, intracellular proteins are usually properly folded. As other eukaryotes, yeasts are also capable of performing most posttranslational processing typical of mammalian cells. However, extracellular proteases and differences in glycosylation in proteins expressed in yeast, compared to those of mammalian cells, limit their use. *N*-glycosylation of proteins produced by yeasts are high-mannose (with more than 3 mannose residues) or hypermannose (more than 6 mannose residues) types, with terminal α-1,3 linkages (***126***). Such forms are very immunogenic to mammals (***127,128***). Moreover, *O*-glycosylation by yeasts con-

tains only mannose residues (***126***). Cell engineering has been utilized for obtaining nonimmunogenic glycoproteins from yeasts. Namely, Chiba et al. (***129***) introduced the gene of an α1,2-mannosidase with an ER retention signal in a *S. cerevisiae* mutant that had disrupted the genes of several mannosyltransferases. Such a manipulation resulted in recombinant and native glycoproteins with the structure M5N2. This structure is not found in glycoproteins produced by *S. cerevisiae* and is the substrate for further processing to yield complex glycans as in mammalian cells.

Unmodified yeasts are suitable for the production of proteins that do not require mammalian-type glycosylation and are resistant to proteases. One of these proteins is insulin, which has been commercially produced in *S. cerevisiae* after enhancing its folding and secretion capacities through genetic engineering (***125***). A promising strategy for enhancing secretion has been published by Tan et al. (***130***), who succeeded in the universal application of a 15-residue secretion signal from bacterial endotoxin. Using such a secretion signal on constructs destined for expression of recombinant proteins both in prokaryotes and eukaryotes, *S. cerevisiae* among them, the model protein was secreted in all cases.

Facultative methylotrophic yeasts, such as *Pichia pastoris*, *P. methanolica*, *Candida boidinii*, and *Pichia angusta* (formerly known as *Hansenula polymorpha*), are hosts with great potential and with various recombinant proteins within reach or already in the market (***126***). Some of these proteins are hepatitis B vaccine, human serum albumin, phytase, and insulin-like growth factor (***126***). Industrial application of methylotrophic yeasts started when they were utilized for single-cell protein production. Very large fermentations of methylotrophic yeasts were performed in the 1970s. As single-cell protein production was not economically attractive, *Pichia pastoris* was proposed as a host for recombinant protein production in the 1980s (***131***). Very high cell densities have been obtained, up to 100 g of dry weight per liter, and also high protein concentrations, up to 1 g/L of secreted recombinant protein (***127***). Additionally, *N*-glycosylation proceeds differently than in *S. cerevisiae*, with hypermannosylation being less elaborate (***132***) and occurring less frequently (***126***). Importantly, *P. pastoris* does not produce the immunogenic terminal α-1,3-linked mannoses (***132***). Similarly to what has been performed in *S. cerevisiae*, Callewaert et al. (***132***) constructed a recombinant *P. pastoris* expressing the α1,2-mannosidase gene with a retention signal that targets the enzymatic activity to the ER-Golgi transit region. As a result, M5N2 glycans were the most common structures attached to the recombinant protein.

A potential disadvantage of *P. pastoris* and *P. methanolica* is that transgenes are placed under the promoter of the alcohol oxidase I (AOX1) gene, which requires methanol to induce gene expression. This has three implications for the process. First, large tanks of flammable methanol are needed in the production facilities, and second, methanol, which is toxic to humans, must be thoroughly removed from the final product. Methanol is also toxic to the cells; thus, a third consideration is that specifically designed methanol feeding strategies must be implemented to guarantee its continuous supply during the induction stage but avoiding its accumulation to inhibitory levels. An alternative is the use of the promoter of the MOX1 gene, which is induced either by methanol or derepressed by glycerol in *P. angusta* (***133***).

Filamentous fungi have been utilized for a long time for the production of a wide variety of substances with various applications. Fungi fermentations at large scales have been performed since the first half of the 20th century, mostly for the production of antibiotics or ascorbic acid (***81***). Fungi can secrete large amounts of homologous proteins (up to 30 g/L), and up to 3 g/L of heterologous proteins, although typically only tens of milligrams per liter are obtained (***134***). Such a difference is a consequence of RNA instability or incorrect processing and of high protease activity (***135***). Recombinant protein concentration has been increased by fusing the gene of interest with genes of fungal origin (***134,135***). Fungi produce proteases; this limits their utility for recombinant protein production. Promoting growth in pellets and controlling pH can reduce protease activity more than fourfold (***136***). Such strategies have been utilized for the commercial production of chymosin. Additionally, many homologous fungal proteins (mostly enzymes) have been engineered to obtain some desirable characteristics not present in the original counterpart.

As in yeasts, filamentous fungi produce high-mannose-type glycans, easily recognized by mammalian lectins; therefore, recombinant proteins intended for therapeutic use and expressed in fungi can be rapidly and inconveniently cleared from blood. Trying to palliate for this inconvenience, Maras et al. (***137***) first demonstrated that glycoproteins from *Trichoderma reesei* could be converted in vitro to mammalian-like hybrid oligosaccharides. Later on, Maras et al. (***138***) expressed in *Trichoderma reesei* the human *N*-acetylglucosaminiltransferase I that transfers an N-acetylglucosamine residue to an α-1,3-linked mannose of the M5N2 oligosaccharide. Efforts like this indicate that mammalian *N*-glycans expressed in filamentous fungi are not far away.

3.3. Animal Cells

Animal cells have been cultured in vitro for more than a hundred years. For a long time they have been used for the production of viruses as vaccines, or for synthesizing endogenous proteins, such as interferon. Their complexity delayed their genetic manipulation to the time when manipulation of bacterial genomes was performed almost routinely. The first recombinant proteins approved for human use were produced in bacteria, but of 33 products approved by the FDA between 1996 and 2000, 21 are produced by animal cells. It is expected that this situation will continue as more proteins with pharmaceutical applications have complex glycosylation that cannot be practically produced in prokaryotes or lower eukaryotes (*see* Chapters 28–33). In the mean time, animal cell culture has become routine, with several reactors operating worldwide at the 10,000-L scale. However, successful recombinant protein production in animal cells had to overcome many hurdles, such as the cellular fragility and the complex nutritional requirements of cells (***93,94,139***). Animal cells require hormones and growth factors that were initially supplied by bovine serum. Possible contamination of the final product with virus or prions, and the difficulty of recovering extracellular proteins from serum-containing media, have resulted in the development of serum-free media that are used for large-scale production.

Gene transfer is a particularly relevant issue in cell culture. The development of mammalian cell culture methodologies included designing a variety of vector systems (*see* **Table 4** for a synthesis of their components) and of gene transfer methods (***140***). A

Table 4
Summary of the Required Elements in a Mammalian Expression Vector

				Transient Expression	Stable expression	Selection markers	
Viral-based	Plasmid-based	Promoter/ enhancer	Locus control Regions[a]	Reporter gene	Chromosomal or episomal	Gene-deficient cells	Cytotoxic drugs
• Adenovirus • Epstein–Barr • Herpes simplex • Papilloma • Polyoma • Retrovirus • SV40 • Vaccinia	• Prokaryotic, eukaryotic, and viral sequences	• Adenovirus inverted terminal repeats (ITR). • Citomegalo virus • Mouse mammary tumor virus • Murine leukemia virus (MuLV) long terminal repeat (LTR) • Rous sarcoma virus (RSV) LTR • SV40 • β-actin • α-fetoprotein • γ-globin/-β-globin • β-interferon • Metallothionein II	• Human β-globin locus • Human adenosine deaminase gene • Human apolipoprotein E/C-1 gene locus • Human T cell receptor α/δ locus • Human CD2 gene • Human S100 β gene • Human growth hormone gene • Human apolipoprotein B gene	• Chloramphenicol Acetyltransferase (CAT) • β-Gal • Firefly luciferase (Luc) • Human placental alkaline phosphatase (AP) • β-glucoronidase (GUS) • Green Fluorescent Protein (GFP)	• BKV-based vectors[b] • Bovine papilloma virus-based vectors[b] • Epstein-Barr virus-based vectors[b]	• Herpes simplex virus thymidine kinase • Dihydrofolate reductase (dhfr) • Hypoxantine guanine phosphoribosyl transferase (hprt) • Adenyl phosphoribosyl transferase (aprt)	• Hygromycin B phosphotransferase (HygB) • Xanthine-guanine phospho ribosyl transferase (XGPRT) • Zeocin (Zeo) • Blasticidin (Bsd) • Aminoglycoside phosphotrasferase (aph)

[a]Shows only a limited selection of the available loci listed in Li et al. (***159***). [b]Some of the most frequently used viral-based episomal vectors (***160***).
Data from **ref. *140***.

recent development in gene transfer is the use of baculovirus vectors in cultured mammalian cells. Baculoviruses used with this objective carry promoters that are efficiently transcribed in mammalian cells, such as those from Rous sarcoma virus or cytomegalovirus. This methodology has been tested (***141***) of rat hepatic stellate cells, showing a 100% efficiency of heterologous gene expression (*lacZ*) using elevated multiplicities of infection (500 plaque-forming units per cell) in an *Autographa californica* multiple-nucleocapsid polyhedrovirus. This report as well as the increasing number of references relating to the use of baculovirus for gene transfer in mammalian cells, both in vitro and in vivo, show the promises of this approach.

Other issues that arise when expressing proteins in mammalian cells can be solved through cell engineering. For example, when large scale production is engaged, the cells suffer metabolic pressures, such as oxygen depletion and toxic metabolite accumulation, which affect final yields. An interesting approach with CHO cells (***142***) consisted of engineering their mRNA translation initiation machinery with the aim of leaving it on, despite the prevalence of stressful conditions derived from large-scale production schemes. Traditional strategies for productivity optimization involve manipulation of cell division as well as cell longevity, supported by the increasing knowledge of cell cycle control (***143***). Such is the case of the manipulation of a myeloma cell line that constitutively expresses a chimeric antibody. The cell line was modified to express, upon induction, an inhibitor of cyclin E-dependent kinase that causes cell cycle arrest. With this manipulation, Watanabe et al. (***144***) arrested cell proliferation, thus preventing accumulation of deleterious metabolites. Additionally, with this operation the yield of a recombinant hybrid antibody was enhanced 4-fold. A somewhat similar strategy was used by Meents et al. (***145***), who arrested *dhfr*-deficient CHO cells in G1 by inducibly expressing the cyclin-dependent kinase inhibitor $p27^{Kip1}$, being able to enhance specific productivities by fivefold (*see* **Subheading 2.2**). Apoptosis represents a major inconvenience in cultures intended for production (***146***), but overexpression of the antiapoptotic gene *bcl2* (***147,148***) leads to sustained growth and therefore sustained protein production (***149,150***).

An important aspect to be considered when expressing recombinant proteins in mammalian cells, and part of the reason that these cells are used as an expression vehicle, is glycosylation. A major drawback that emerges from altering the glycosylation machinery in vivo is the resulting heterogeneity of products (***151***), given the variety of pathways that can be followed. In spite of this, and given the subtle differences that exist between glycans obtained in commonly used mammalian cell lines and those associated with glycoproteins synthesized in human cells, cloning glycosyl-transferases into common mammalian cell lines has proved useful for the expression of humanized *N*-glycoproteins (***148***) and *O*-glycoproteins (***152***).

A rather new and exciting application of mammalian cell culture is gene-function analysis (***153***). The key tool for this application is RNA interference (RNAi), which occurs by sequence-specific gene silencing initiated by double-stranded RNA (dsRNA) homologous to the gene to be silenced. The mediators for mRNA degradation are small 21 to 22 nucleotide-interfering RNAs (siRNAs) that result from the enzymatic activity of dicer, a cellular ribonuclease III. This specific process seems to have emerged as a defense against aberrant or unwanted gene expression (***154***). Although somehow differently, this

phenomenon also silences genes in mammalian cells, and has been reported in neurogenesis and neuronal differentiation studies (***155)***. Gene silencing was achieved in cultured mouse P19 cells by means of synthetic duplex RNAs as well as with hairpin siRNAs. Hamada et al. ***(156)*** proved the system useful in mammalian cells by targeting mRNA of Jun dimerization protein expressed in mouse RAW264.7 and NIH3T3 cells with both duplex RNAs and the sense strand of the synthetic siRNA. RNA silencing can become a powerful technique for improving recombinant protein production.

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