Overview of Protein Expression by Mammalian Cells

In recent years, mammalian cells have been used in the production of recombinant proteins, antibodies, viruses, viral-subunit proteins, and gene-therapy vectors. In addition to being used in commercial biotechnology, mammalian cell systems have served as a means for examining fundamental aspects of gene replication, transcription, translation, and post-translational protein processing.

The availability of transformable cell lines, along with viral and plasmid-based mammalian-cell vector systems, has provided tools through which important aspects of mammalian gene function can be investigated. The following are typical uses for mammalian expression systems:

1. verification of a cloned gene product;
2. analysis of the effects of protein expression on cell physiology;
3. production and isolation of genes from cDNA libraries;
4. production of correctly folded and glycosylated proteins for assessment of biological activity in both in vitro and in vivo systems;
5. production of suitable quantities of proteins and glycoproteins for structural characterization of protein and carbohydrate moieties;
6. production of important clinically active viral surface antigens—e.g., prehepatitis B virus surface antigen (preS2 HBVsAg)—as well as therapeutic proteins—e.g., β-interferon, tissue plasminogen activator (tPA), erythropoietin (EPO), and Factor VIII; and
7. production of monoclonal antibodies.

Important features of mammalian cells include their ability to perform post-translational modifications and to secrete glycoproteins that are correctly folded and contain complex antennary oligosaccharides with terminal sialic acid. These covalent modifications may modulate the clinical efficacy of the protein (e.g., circulatory half-life and biospecificity) or result in properties that are of interest for biochemical characterization—e.g., with respect to structural stabilization, functional groups, and biological role. Mammalian-produced proteins are quality-controlled through a process whereby the progress of incompletely folded, misassembled, and unassembled proteins into the secretory pathway is selectively inhibited (Hurtley and Helenius, 1989). The correctly processed material progresses and is generally secreted as fully active protein. Another important feature of mammalian cell expression has been the ability to provide proteins bearing sialyl glycoforms closely resembling glycoproteins produced by humans. In contrast, *E. coli* does not possess any glycosylation machinery and yeast tends to produce hypermannose-type glycosylation. The yeast-produced glycoproteins are rapidly cleared from the circulation by the liver and also may induce adverse immunogenic responses in humans.

The post-translational modifications performed by mammalian cells include the proteolytic processing of the propeptide (Neurath, 1989) as well as glycosylation and carbohydrate trimming (Hirschberg and Snider, 1987). Other modifications may include γ carboxylation of glutamic acid residues (Suttie, 1985; Kaufman et al., 1986a); hydroxylation of aspartic acid and asparagine residues (Stenflo, 1989); sulfation of tyrosine residues (Baeuerle and Huttner, 1987); phosphorylation of proteins through cell receptor–protein interaction (Myers et al., 1994); fatty acid acylation (McIlhinney, 1990); and correct assembly of multimeric proteins. In contrast to what happens in mammalian cells, the production of intracellular recombinant proteins in *E. coli* leads to accumulation of the protein in reduced form, often as insoluble refractile bodies requiring subsequent denaturation and refolding.

This unit will briefly review the stages involved in protein production in mammalian cells using the stable-expression approach as outlined in Figure 5.9.1. Discussion will include choice of cell type, transfection of the host cells, methods for selection and amplification of transformants, and growth of cells at appropriate scale for protein production. Since post-transcriptional modification and intracellular protein transportation are important features of recombinant-protein production in mammalian cells, some description of these mechanisms is included.

CHOICE OF MAMMALIAN CELL HOST

Although a variety of mammalian cell hosts are available for protein production, only a small number have emerged as systems of choice for production of proteins to be used clinically. The most common of these cell hosts

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are summarized in Table 5.9.1. The narrowing down of choices is largely due to the need for cell lines that: (1) are capable of continuous growth; (2) can be grown in suspension (in bioreactors); (3) have low risk of adventitious infection by potentially pathogenic viruses; (4) have genetic stability; and (5) can be readily characterized with respect to karyology, morphology, isoenzymes, and gene copy number. The existence of a variety of host-cell systems, the availability of viral or cDNA-based vectors, and the possibility of either stable or transient expression requires that the prospective user define an expression strategy based on ultimate goals. When the researcher's objectives require <1 mg of protein, transient expression in COS-7 cells is the relevant route. Transient expression in the COS-cell and vaccinia systems has been recently reviewed (Moss and Earl, 1991; Aruffo, 1997), and detailed protocols for construction of suitable vectors and protein expression by these systems can be found in those articles.

In transient expression a burst of production occurs in the host cell and is usually accompa-

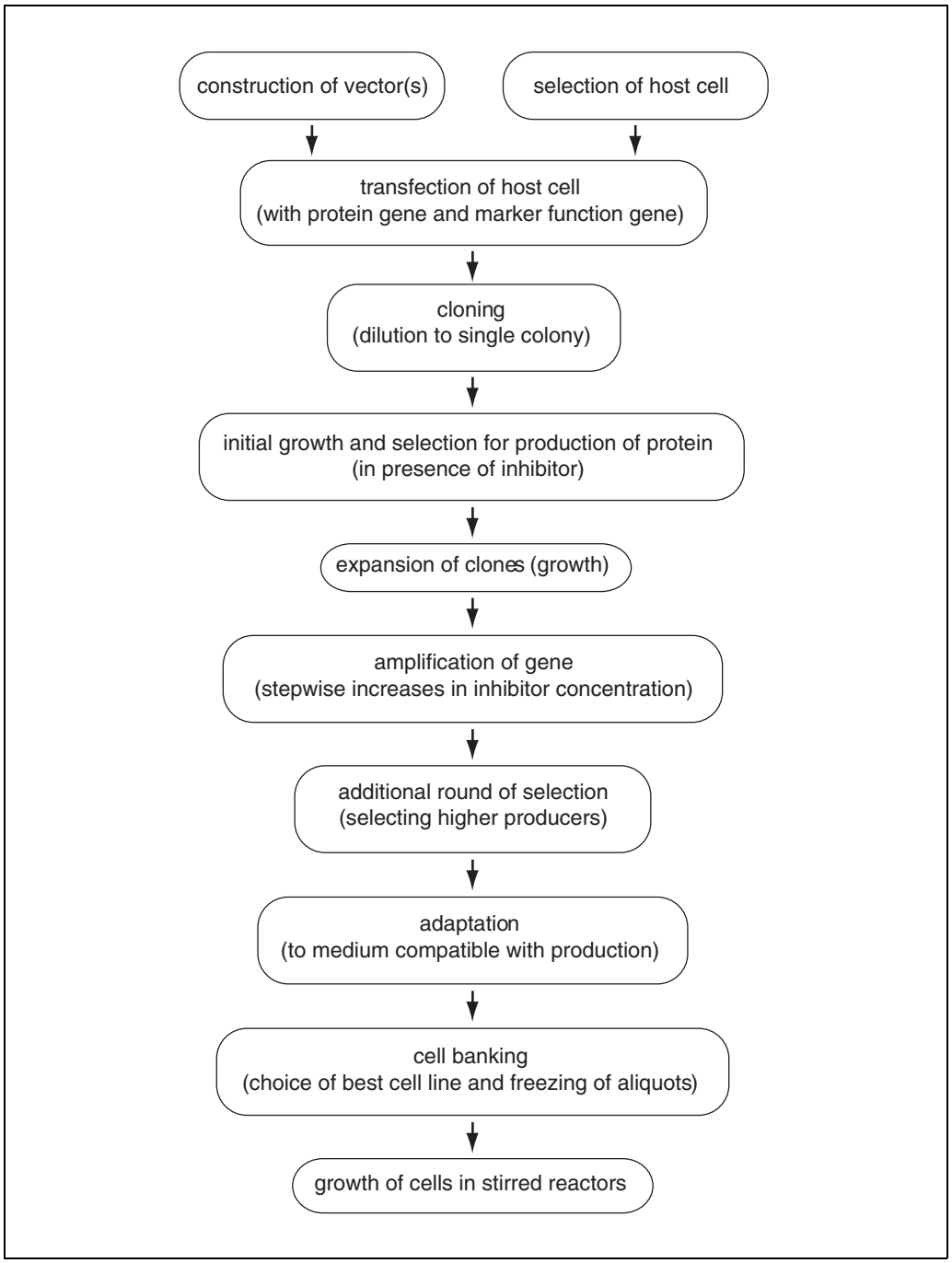

Figure 5.9.1 Flow chart of activities in the development of a mammalian-cell expression system.

Table 5.9.1 Common Mammalian Cell Hosts

Cell line	Description	Growth	Utility	Source[a]
Human				
Namalwa	Burkitt's lymphoma–transformed lymphoblastoid cell	Large-scale suspension	Production of α interferon (e.g., by Burroughs Wellcome)	ATCC #CRL-1432
HeLa	Aneuploid cervical carcinoma cell	Small-scale suspension	Production of small quantities of research material (few mg)	ATCC #CCL-2
293	Transformed kidney cell	Small-scale suspension	Production of small quantities of research material (few mg)	ATCC #CCL-1573
WI-38	Human diploid normal embryonic lung cell	Attachment only	Host for virus production	ATCC #CCL-75
MRC-5	Human diploid normal embryonic lung cell	Attachment only	Hardier host for virus production—e.g., hepatitis A	ATCC #CCL-171
HepG2	Liver carcinoma transformed cell	Attachment	Small-scale evaluation of expression—e.g., HBVsAg	ATCC #HB-8065
Rodent				
3T3	Swiss mouse embryo fibroblast	Attachment	Used in testing transforming agents, expression evaluation	ATCC #CCL-92
L-929	Normal connective tissue fibroblasts	Attachment	Small-scale evaluations of expression	ATCC #CCL-1
Myeloma (e.g., NS/O)	Many types	Large-scale suspension	Monoclonal antibody production	ATCC (and commercial sources)
BHK-21	Baby hamster kidney cell	Large-scale suspension	Host for virus production or for stable gene integration	ATCC #CCL-10
CHO-K1	Chinese hamster ovary cell	Large-scale suspension	Used with glutamine synthetase system	ATCC #CCL-61
CHO DG44	Chinese hamster ovary cell	Large-scale suspension	Host for DHFR coamplification	L. Chasin[b]
CHO DXB11	Chinese hamster ovary cell	Large-scale suspension	Preferred host for DHFR coamplification	L. Chasin[b]
Monkey				
COS-7	Transformed African green monkey kidney cell	Small-scale attachment	Transient expression host	ATCC #CCL-1651
Vero	Normal African green monkey kidney cell	Large-scale attachment	Production of viruses	ATCC #CCL-81

[a]Abbreviation: ATCC, American Type Culture Collection (see *SUPPLIERS APPENDIX*).

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nied by death and rapid lysis of the cell. This presents the purification scientist with the challenge of fishing out the protein of interest, which may be present at ~5 µg/ml, from a soup of lysed cellular protein, nucleic acids, and viral particles. The yield of product during purification may be low as a result of the low titer and starting purity; however, when only small quantities of protein are required, transient expression in COS cells or the vaccinia system is a quick and suitable system to employ.

For production of larger quantities of protein, stable expression must be used because of the difficulty in scaling up transient expression into a bioreactor system. Stable expression in Chinese hamster ovary (CHO) cells is described in *UNIT 5.10*.

Some cell lines have successfully been used as hosts in production of viruses and useful proteins. These include the human embryonic lung cell line MRC-5 and the normal embryonic cell line WI-38. These cells are attachment growth–dependent and have a finite lifespan of ~50 generations, after which time the cells enter a senescent phase and begin to die.

Cell lines that have undergone transformation by virus or that have experienced alteration of chromosomes can exhibit a capacity for infinite growth as well as an ability to grow in suspension. Examples of such cell lines are HeLa cells from a human cervical cancer and Namalwa cells from a human lymphoma. There has been some reluctance to use these cell lines in production of clinical agents because of the possibility of transfer of tumorigenic agents to the product. For production of larger amounts of recombinant protein, stable expression is required and has been primarily performed in CHO cells, baby hamster kidney (BHK-21) cells, or myeloma cells (e.g., NS/O). These cell lines are capable of indefinite growth on a large scale and are suitable hosts for stable integration of heterologous DNA.

Stable expression results from integration into the host-cell genome of the gene for the expression of the heterologous protein. The integrated gene is transcribed efficiently and the protein is expressed persistently over many generations by the host cell. A stable producer-cell line typically provides ~1 to 10 mg of secreted protein per 10^9 cells per day (specific productivity). For monoclonal antibody production, productivity levels of 35 to 100 mg per 10^9 viable cells per day in myeloma cells (Bebbington et al., 1992; Shitara et al., 1994) and 15 to 110 mg per 10^9 viable cells per day in CHO cells (Page and Sydenham, 1991) are obtainable. Such levels may allow secreted-antibody titers of 1 to 1.5 mg/liter to be achieved in optimized large-scale systems; hence these systems have been popular in biotechnology. Where protein is accumulated intracellularly, it may reach little more than 0.1% to 0.5% of total cell protein. Thus, the focus has tended to be on secretion of protein where significant amounts of product are obtainable.

The obvious drawback associated with stable expression in mammalian cells is the time required to obtain stable productive cell banks (months) versus that required to obtain large amounts of non-post-translationally modified proteins in *E. coli* or large quantities of high-mannose-containing glycoprotein using yeast and insect cells (2 to 3 weeks). The advantages of obtaining the more complex proteins from mammalian cells include the ability to understand how these molecules interact in biological models both in vitro and in vivo. Using mammalian systems, much knowledge has been gained in the understanding of post-translational modification, protein quality control, and protein translocation. This knowledge ultimately facilitates improved expression in mammalian hosts.

Stable expression requires the transfer of the foreign DNA, along with the relevant DNA-based signals for transcription by RNA polymerase II, into the chromosomal DNA of the host cell (e.g., CHO). A suitable plasmid vector is required to carry the functions through to the host DNA. Vectors are commercially available from a variety of vendors—e.g., Invitrogen, Clontech, and Promega (see *SUPPLIERS APPENDIX*). A large variety of vectors are described in Pouwels et al. (1988). Suitable replicons contain 5 to 10 kb of DNA and usually are designed to contain phenotypic markers for selection in *E. coli* (e.g., Ap^r) and CHO cells ($DHFR^+$). Other features of appropriate plasmids include a promoter-distal cloning site along with other cloning sites; a replication origin for *E. coli*; a polyadenylation sequence from SV40 or bovine growth hormone; a eukaryotic origin of replication (e.g., SV40 or oriP); the gene for expression of the protein in a site proximal to the promoter; and a promoter and associated elements. Commonly used constitutive promoters for CHO cell applications are the SV40 early promoter (Moreau et al., 1981; Neuhaus et al., 1984), the adenovirus late promoter (McKnight and Tjian, 1986), and the cytomegalovirus (CMV) early promoter (Boshart et al., 1985). Transcriptional enhancers such as the human CMV enhancer (Boshart et al.,

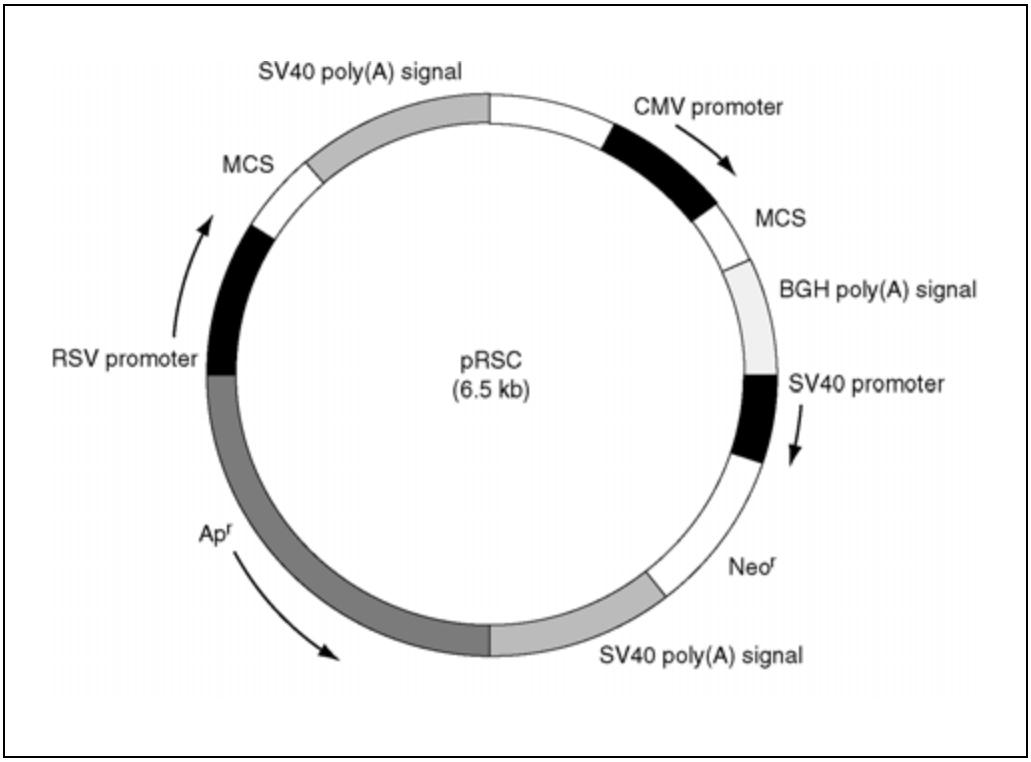

Figure 5.9.2 Representation of a mammalian expression vector based on the vector pRSC, published by Tsang et al. (1997); adapted with permission. The plasmid has two multiple cloning sites (MCSs) and three promoters. One promoter drives the neomycin-resistance protein while the remaining two MCSs have specific promoters and can be used to incorporate an amplification marker (DHFR) and a gene for the protein of interest.

1985) can enhance transcriptional activity by 10- to 100-fold and may be incorporated into the vector. These features are illustrated in Figure 5.9.2.

CHO cells have been used extensively as a host for stable expression of proteins. A number of CHO mutants have been developed that provide the user with tools to examine the synthesis of DNA, RNA, and protein as well as protein secretion, protein glycosylation, and intermediary metabolism. The most popular CHO sublines (also see Urlaub and Chasin, 1980) are CHO DXB11 (*dhfr*$^+$/*dhfr*$^-$) and CHO DG44 (*dhfr*$^-$/*dhfr*$^-$). The DXB11 cell was derived from CHO-K1 in 1978 by Lawrence Chasin. The CHO-K1 cells were originally derived in the 1950s, and the cells that were used in Chasin's laboratory for the development of the DXB11 line were obtained from Ted Puck and Fa-ten Kao at the Eleanor Roosevelt Cancer Institute in Denver in 1970. The DG44 cell line contains a double mutation in the *dhfr* genes and is not capable of natural reversion to the *dhfr*$^+$ phenotype. The DG44 cell line was derived from CHO pro3$^-$ cells by Chasin in 1982. The CHO pro3$^-$ was derived from the cell line established in the 1950s and is sometimes referred to as CHO Toronto, because it was used extensively in that city by Louis Siminivitch and colleagues. Both these cell lines can be obtained from Dr. Lawrence Chasin of Columbia University (e-mail: *lac2@columbia.edu*). Other mutants have been produced, and a brief list is included in Wirth and Hauser (1993).

TRANSFECTION, SELECTION, AND AMPLIFICATION

For introduction of genetic material into mammalian cells, three types of vectors are available—viruses, plasmid vectors with a replicon for animal cells, and plasmid vectors without a replicon for animal cells. Most viruses that replicate in mammalian cells ultimately cause death and lysis of the host cell, so expression of virus-borne genes is transient and can only be obtained over a short period prior to lysis. Retroviruses are capable of integrating genetic material into the chromosome of host cells—i.e., stable transformation. As with viruses, the use of plasmid vectors can result in either stable or transient expression of a gene product; the plasmid vectors without a mam-

malian cell replicon would require stable integration for expression of gene product.

Stable production of foreign proteins by mammalian cells requires use of a host cell line that is capable of incorporating the heterologous-gene DNA into a chromosome. In addition to the need for successful stable integration, a selection method is needed to fish out the stable transformants from the large population of cells that may contain unstable, intracellular heterologous gene copies. This necessitates cotransfection of a gene conferring some unique phenotypic property. Resistance to cytotoxic drugs is the most frequently applied method for selection of stable transformants. Normally, a gene for recessive drug resistance would be cotransfected into a host cell deficient in that particular activity. The most common approach is the use of dihydrofolate reductase (DHFR), which can be used as a selectable marker in DHFR-deficient CHO cells (Kaufman and Sharp, 1982). Other common transfectable selectable amplification markers that can be used with CHO cells are adenosine deaminase (in presence of inhibitors of ADA or cytotoxic levels of adenine or its analogs) in CHO DXB11 ($DHFR^-$; Yeung et al., 1983; Kaufman et al., 1986b); carbamyl phosphate synthetase–aspartate transcarbamylase–dihydroorotase in CHO UrdA mutant cells (de Saint Vincent et al., 1981); asparagine synthetase in CHO N3 (AS^-) cells (Cartier and Stanners, 1990); glutamine synthetase in any CHO cell (Bebbington and Hentschel, 1987); ornithine decarboxylase in CHO C55.7 (ODC^-; Chiang and McConlogue, 1988); multiple drug resistance in any CHO cell (Kane et al., 1989); and a mutant DHFR (Leu 22 changed to Arg 22) which can be used in any CHO cell host (Simonsen and Levinson, 1983). See Mortensen et al. (1997) for additional discussion of the use of selectable markers with mammalian cells.

It has been reported that a highly variable but typically large fraction (20% to 100%) of CHO clones established through calcium phosphate– or lipofection-mediated cotransfections (of *dhfr* and heterologous protein genes) coexpressed the dual-transfected expression vectors (Wurm and Petropoulos, 1994). It is likely that integration occurs at random sites. Given that the mammalian-cell genome is large ($>10^9$ bp), has 20% to 30% of the DNA as single-copy, and has only 0.1% of the genomic DNA containing coding sequences, it is critical to exert selective pressure to screen out the large number of nonproductive clones. Further amplification in the presence of selective pressure is necessary to obtain clones in which multiple copies of the heterologous gene are obtained through coamplification with the *dhfr* or other selectable marker gene. In this respect it is wise to screen for high producers at moderate levels of methotrexate (<1 μM) to prevent enrichment of high-copy-number but unstable integrands. Stability is assessed through determination of productivity after extended growth or passaging in the absence of selective pressure in complete medium.

Following transfection of cells using calcium phosphate precipitation or lipofection (Kaufman, 1990a), ~5% to 50% of the cells in the population acquire DNA and transiently express the encoded protein over a period of days before unstable, nonintegrated DNA is lost from the cells. A smaller portion of the cells will integrate the DNA chromosomally, resulting in stable protein expression. Following selection of these stable-expressing cells by application of selective pressure, less than or equal to approximately 0.1% of the original population will express the foreign protein. It is thus imperative to use selective pressure in the production of the early stable-expressing clones to prevent overgrowth with nonproducing cells. For CHO $dhfr^-$ host–based production, the cells are grown in medium deficient in nucleosides (e.g., α-MEM medium from Life Technologies), containing serum that has been dialyzed (to remove low-molecular-weight nucleosides such as hypoxanthine), and supplemented with 0.1 to 1.0 μM methotrexate. In deficient medium, the cell's requirement for purine and pyrimidine nucleosides is satisfied by the action of heterologous dihydrofolate reductase, which converts folate from the medium to tetrahydrofolate (FH_4). FH_4 and serine are then used by cellular serine hydroxymethyl transferase to provide methylene-FH_4 which can go on to donate C1 units in the reactions required for inosinic acid and thymidine production. Cells that acquire the capacity to express *DHFR* can be selected by medium deficient in nucleosides. Stepwise increases in the methotrexate concentration with increased passaging in deficient medium will select for cells containing high copy number of *DHFR* (Kaufman, 1990b).

PROTEIN TRANSLATION, QUALITY CONTROL, AND COVALENT MODIFICATION

Mammalian cells are complex factories with large numbers of compartments, each with specialized functions. Synthesis of proteins begins

Table 5.9.2 Protein Translocation and Post-Translational Modification Signals

Protein-based signal sequence	Related cell function	Example	Reference
$KDEL_{COOH}$	Retention in ER	BiP, protein disulfide isomerase, prolyl isomerase	Pelham and Bienz (1982)
Transmembrane sequences	Golgi retention	Coronavirus matrix protein	Mayer et al. (1988); Machamer and Rose (1987)
Mannose-6-phosphate	Added in Golgi for transport to lysosome	Lysosomal hydrolases	Reitman and Kornberg (1981)
SKL_{COOH}	Peroxisomes	Luciferase acyl coenzyme A oxidase	Keller et al. (1987); Miyazawa et al. (1989)
Leader peptide	Mitochondria	CoXIV (cytochrome *c* oxidase subunit IV)	Hurt et al. (1984)
RRNRRRRW (other specific signals exist, e.g., for p53 and SV40 late T antigen)	Nucleus	Rev	Malim et al. (1989)
Asn-X-Ser(Thr)	N-linked glycosylation in ER (dolichol precursor addition)	Many normal secreted and recombinant proteins	Hirschberg and Snider (1987)
O-Ser/Thr	Golgi-based O-glycosylation	Includes some recombinant proteins—e.g., IL-2, tPA	Hart et al. (1988); Conradt et al. (1990)
Cys (ER) Cys (Golgi)	Palmitoylation Myristoylation	Membrane anchoring—e.g., HBV preS1	McIlhinney (1990)
Tyr (Golgi)	Sulfation	Biological function	Rosenquist and Nicholas (1993); Han and Martinage (1992)
Tyr (cell-surface kinases)	Phosphorylation	Signal transduction and some viral proteins—e.g., HBV core	Myers et al. (1994); Albin and Robinson (1980); Aitken (1996)
Asn, Asp (Golgi)	Hydroxylation	Biological function	Stenflo et al. (1989)
Glu (Golgi)	γ-carboxyglutamate	Calcium binding—e.g., blood-cascade factors	Suttie (1985); Kaufman et al. (1986a)

in the nucleus with transcription and progresses to the cytoplasm, where translation and translocation into the endoplasmic reticulum (ER) occurs. The protein can contain sequences (motifs) that will order specific post-translational modifications as the protein progresses through the ER into the Golgi and before reaching the final destination either as a secreted protein or as a specialized resident cellular protein (see Table 5.9.2). In general, initiation of gene-transcription is the rate-limiting step. The rate of transcription elongation is constant until the movement of RNA polymerase II is stopped by termination signals. It follows that a highly expressed gene would be distinguished by the presence of a high density of polymerase molecules, resulting from high-frequency initiation.

For recombinant proteins that are destined for secretion, the translocation of expressed protein follows the so-called constitutive route (Walter and Lingappa, 1986), which is shown in part in Figure 5.9.3. A signal-recognition particle (SRP) binds to the signal sequence of the growing peptide when it protrudes into the cytoplasm, thereby halting further synthesis of the protein until the ribosome-SRP complex has bound to the docking protein (SRP receptor) at the ER membrane. Protein synthesis then continues and the peptide chain progresses into the lumen of the ER. At this point the amino-terminal signal sequence is cleaved off. Signal peptides encompass 13 to 30 amino acids. They usually have a net positive charge at the N terminus, a core of nine or more hydrophobic amino acids, and a cleavage site (Von Heijne, 1983, 1984). The tissue plasminogen activator

(tPA) leader peptide has been used for efficient translocation (Burke et al., 1986). The protein is folded and glycosylation is initiated in the ER. Detailed discussion of the intracellular factors involved in protein translocation are given in a number of excellent reviews (Gething and Sambrook, 1992; Rothman and Orci, 1992; Wirth and Hauser, 1993; Rothman, 1994).

The protein begins to fold cotranslationally in the ER. The ER provides an environment optimized for protein folding and is distinguished by a relatively high concentration of calcium (Baumann et al., 1991). The folding and stability of some proteins is calcium-dependent (Lodish and Kong, 1990). Another significant feature of the ER is that its redox potential is sufficiently high to enable disulfide-bond formation (Hwang et al., 1992). It appears

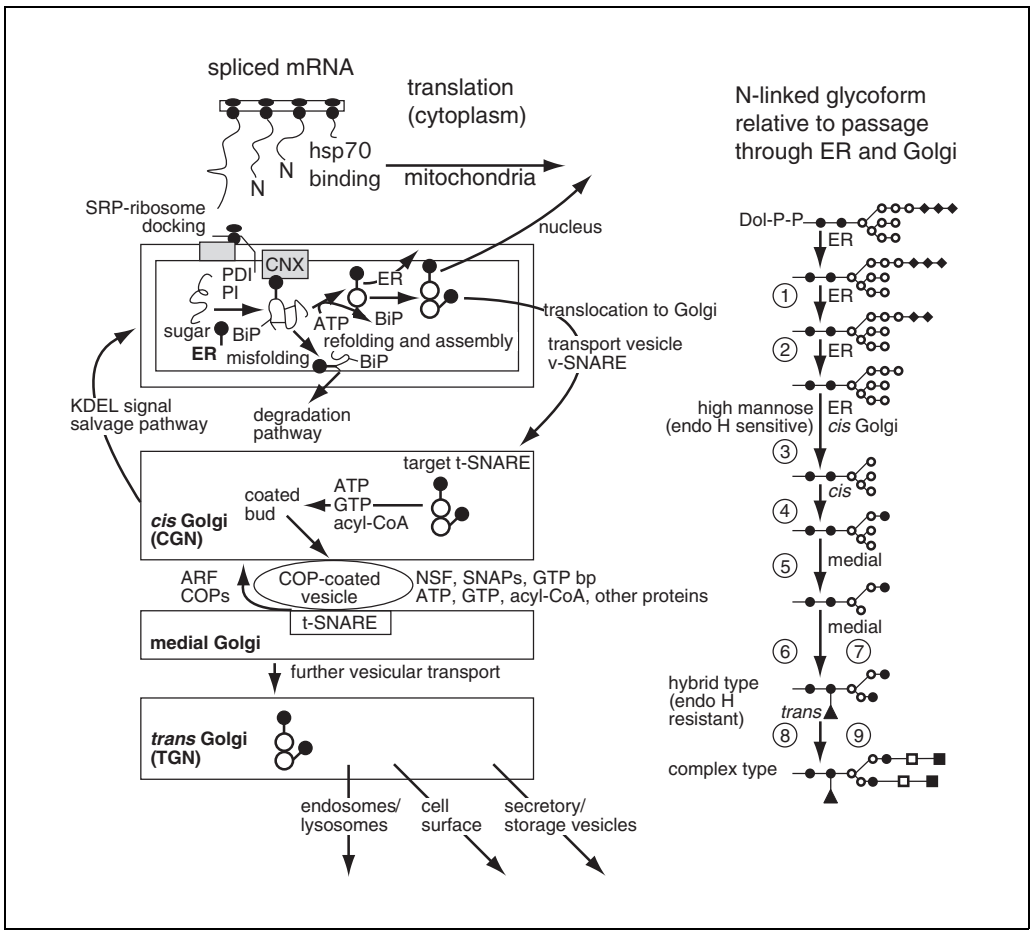

Figure 5.9.3 Intracellular protein translation and cisternal transport. Cytoplasmic translation results in a nascent polypeptide that is chaperoned by the hsp70 protein, which may direct the ribosome-peptide complex to the mitochondrion or the ER upon N-terminal peptide signaling. A signal-recognition particle (SRP) binds to the signal sequence, halting translation until it has docked at the SRP receptor. The hsp70 protein maintains the protein in a translocation-competent state, enabling membrane protrusion. Inside the ER the protein undergoes refolding assisted by protein disulfide isomerase (PDI), BiP, and calnexin (CXN). Initial N-glycosylation takes place cotranslationally in the ER. Misfolded protein, associated with BiP, is retained and eventually degraded. Transport of folded, assembled protein between the ER and Golgi cisternae occurs through a series consisting of budding, vesicle formation, and specific binding to target cisternae surfaces, through to the *trans* Golgi (TGN), where distribution to particular destinations occurs. **Abbreviations:** ARF, ADP-ribosylation factor; COPs, a family of coat proteins; Dol, dolichol; endo H, endoglycosidase H; GTP bp, GTP binding protein; NSF, NEM-sensitive cytosolic factor (identical to yeast sec 18p); SNAPs, three soluble NSF attachment proteins; v-SNARE and t-SNARE, SNAP receptors. **Symbols:** solid circles, N-acetyl glucosamine; open circles, mannose; solid diamonds, glucose; open squares, galactose; solid squares, sialic acid; solid triangles, fucose; circled 1, α-glucosidase I; circled 2, α-glucosidase II; circled 3, α-1,2-mannosidase; circled 4, GlcNAc transferase I; circled 5, α-mannosidase II; circled 6, GlcNAc transferase II; circled 7, α-1,6-fucosyl transferase; circled 8, β-1,4-galactose transferase; and circled 9, α-2,3-sialyl transferase.

that glutathione is the redox buffer and that the redox potential of the ER is 20 to 100 times more oxidizing than that of the cytosol as indicated by the GSH/GSSG ratios (between 1:1 and 1:3 in the ER as compared to between 30:1 and 100:1 in the cytosol; Hwang et al., 1992). The ER contains soluble folding enzymes such as protein disulfide isomerase (PDI) and prolyl isomerase, which serve to aid the refolding process. Another group of proteins known as chaperones (Ellis and Hemmingsen, 1989) are present in the ER and appear to assist in protein folding and to ensure that incorrectly folded protein is not released from the ER, thus playing a quality-assurance role for newly synthesized protein in the cell. The best-studied chaperone is the glucose response protein BiP (or GRP 78), a member of the hsp70 family of proteins (Munro and Pelham, 1987; Hendershot et al., 1988). An ER integral membrane protein called calnexin (Bergeron et al., 1994) also appears to function in quality control of protein folding in the ER and affects the retention of incorrectly folded protein. Calnexin is unrelated to the heat-shock chaperone Hsp60, Hsp70, and Hsp90 families.

As the protein is folding in the ER, N-linked oligosaccharides are added to the nascent chain via dolichol phosphate. Intramolecular disulfide bonds are formed with the assistance of protein disulfide isomerase (PDI), and the molecular chaperone BiP/GRP78 associates with the refolding peptide chain. Multiple cycles of BiP binding and release occur in a process that requires ATP. Protein folding occurs with high efficiency and with a half-time on the order of 1 min. Pulse-chase studies of hepatitis B surface antigen (HBVsAg) secretion in mouse fibroblast cells (Huovila et al., 1992) showed that the formation of HBVsAg dimers in the ER occurred rapidly, within 2 min, while formation of higher-order oligomers occurred with a half-time of 2 hr. The study also provided evidence that the rate-limiting step was likely to be passage from the ER into the Golgi, as mature intracellular glycosylated HBV was not detected. The higher-order assembly of subunits or assembly of multimeric proteins begins in the ER and is likely completed in the so-called 15° compartment (named because of accumulation of molecules there at low temperature). This appeared to be the case for HBVsAg (Huovila et al., 1992). When a monomeric subunit is extensively folded, the BiP dissociates, and for multimeric proteins such as viral membrane proteins, subunit-subunit recognition and assembly occurs prior to transport into the Golgi complex (Doms et al., 1993). Only correctly folded protein progresses from the ER to the Golgi apparatus. Protein may be misfolded as a result of inefficient folding, mutations, heat shock, energy depletion, or addition of exogenous reductants such as dithiothreitol. These misfolded proteins usually form aggregates and typically display permanent and stable association with BiP. Aggregates are eventually degraded unless they are salvaged through a pathway that facilitates refolding from the misfolded protein species under specific conditions—e.g., altered temperature for temperature-sensitive folding mutants or where the ER environment is experimentally altered.

Proteins that have successfully folded in the ER are destined for secretion to the plasma membrane or the intracellular compartments, including the ER itself. Proteins destined to be retained in the ER possess the C-terminal signal sequence KDEL (Munro and Pelham, 1987). Proteins containing the KDEL signal that escape the ER are efficiently salvaged from the Golgi through a divalent cation–dependent receptor-based salvage pathway (Rothman and Orci, 1992). Proteins destined to be integrated into the ER membrane have a signal consisting of two lysine residues separated by three and four or five amino acids apart from the C terminus (Jackson et al., 1990). Signals for Golgi retention appear to involve histidine and cysteine within the membrane-spanning domain (Aoki et al., 1992).

N-linked oligosaccharide assembly begins in the ER compartment (Kornfeld and Kornfeld, 1985). In brief, N-linked glycosylation begins with synthesis of a lipid-linked oligosaccharide moiety—$Glc_3Man_9GlcNAc_2$-P-P-dolichol—which is transferred to the nascent peptide chain at the relevant Asn-X-Ser/Thr sequon site (i.e., peptide sequence recognition site). Truncations of the initial oligosaccharide structure follow this initial step in the ER. The folded and primary-glycosylated protein is transported to the Golgi, where maturation of the carbohydrate chains takes place. O-glycosylation is thought to occur after the N-glycosylation in the ER or the early part of the Golgi.

Differences in glycosylation of a given protein are a result of divergence in the Golgi-based processing of the oligosaccharides in different cell types (Jenkins et al., 1996). The human lymphoblastoid Namalwa cell line performs O- and N-linked glycosylation of recombinant tPA efficiently to produce human-type glycosylation characteristics (Okamoto et al., 1991; Khan et al., 1995). Most CHO cell lines

used in production of recombinant proteins do not possess active α1,3-galactosyltransferase, although the gene is present in these cells, which make low levels of galactosamine neuraminic acid. CHO and BHK cells also lack a functional α2,6-sialyltransferase enzyme and synthesize exclusively α2,3–linked terminal sialic acids via α2,3-sialyltransferase, in contrast to human cell lines, which contain both enzymes (Lee et al., 1989). In CHO cells the dominant sialic acid found is N-acetyl neuraminic acid.

Transport of the protein through the secretory and endocytic pathways is mediated by small vesicles that bud off from one compartment and fuse to the next compartment (Rothman and Orci, 1992). The *cis* Golgi stack (CGN) and *trans* Golgi stack (TGN) are defined areas of the entire Golgi network. The CGN and TGN constitute the entry and exit faces, respectively, and are involved in sorting and distribution of the protein. The CGN includes the KDEL salvage pathway for ER-based proteins. The TGN is where proteins with differing final destinations—e.g., lysosomes, secretory vesicles, and plasma membrane—diverge. The Golgi consists of stacks of cisternae where carbohydrate-chain trimming and elongation as well as other covalent modifications occur. The mechanism of flow through the Golgi cisternae appears to involve assembly of coated vesicles, which then transfer to the next compartment with concomitant loss of the coat. The loss of coat is linked to GTP-dependent hydrolysis, while subsequent fusion at the destination cisternae is ATP-dependent and also involves an N-ethylmaleimide (NEM)-sensitive enzyme, Sec18p. The specificity of vesicle targeting involves a docking mechanism based on binding of soluble NEM-sensitive attachment protein (SNAP) to a receptor designated as SNARE. Heterogeneity in the SNARE family allows for specialization and the ability to direct a given protein in the appropriate direction by selective fusion to progressive cisternae and ultimately the destination membrane.

Although it is usually desirable to obtain secretion of protein out of the cell prior to purification, in some cases the goal may be to express a protein receptor that would be transported to the cell-surface membrane. This was described for the production of the erythropoietin EPO receptor (EPO-R) in transfected hematopoietic cells (Hilton et al., 1995). The polypeptide was mutated to improve EPO-R refolding in the ER and increase cell-surface expression. The protein was obtained as a crude extract from the cell culture medium following the completion of the cell culture process. The glycoprotein may then be purified for further physicochemical analysis or testing in biological assays and animal studies.

Some proteins are not normally secreted by mammalian cells. This is the case for ER-resident proteins such as BiP, prolyl isomerase, and protein disulfide isomerase, which contain the C-terminal ER-retention peptide sequence KDEL. Also, a number of proteins including receptors and signal-transduction proteins are expressed and retained in the cell or on the cell surface. In cases where specific inhibitors of glycosylation enzymes are added to the culture medium, proteins that are normally secreted may be poorly secreted and thus accumulate in the ER or Golgi of the cell (Hickman and Kornfeld, 1978). Release of these intracellular or surface proteins requires disruption of the cell using a method capable of breaking the cell membrane (homogenization); in cases where membrane association is expected, nonionic detergents at 0.1% to 0.5% (v/v) are used in the lysis buffer.

Covalent modifications of amino acids by mammalian cells are numerous and to some extent may be a feature of a particular cell type. For example the γ carboxylation of factor VIII, which is essential for clotting-cascade activity, can be accomplished in the BHK and human 293 cell lines. This microsome-based, vitamin K–dependent modification was nonfunctional in CHO cells that had been transformed with complementing genes for carboxylation (Wirth and Hauser, 1993). Other processing events occur during passage of the protein from the ER to the Golgi. These involve removal of certain residues from the carbohydrate chains—e.g., glucose and mannose trimming. Other sugars are added to the carbohydrate chains as the protein passes through the medial and *trans* Golgi—e.g., addition of *N*-acetylglucosamine, galactose-*N*-acetate, fucose, galactose, and sialic acid. O-linked glycosylation is important for stabilization of some proteins—e.g., low-density-lipoprotein (LDL) receptor (Kingsley et al., 1986).

GROWTH OF MAMMALIAN CELLS FOR PROTEIN EXPRESSION

Mammalian cell culture media are complex in comparison to the simple defined media used for propagation of bacteria and yeast. This complexity stems from the need to satisfy the many functions required for normal growth and

metabolism of mammalian cells (Bettger and McKeehan, 1986). Nutrients must satisfy the requirements for growth, maintenance of the cell, and expression of products. Additional specific growth factors such as insulin or insulin-like growth factor (IGF) are usually required. Minimal nutritional requirements were first defined in the 1950s (Eagle, 1955). These early studies showed that thirteen amino acids (Arg, Cys, Gln, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, Tyr, and Val), eight vitamins (biotin, folic acid, niacinamide, pantothenic acid, pyridoxine, riboflavin, and thiamine), and various salts and minerals (Na, K, Ca, Mg, Cl, PO_4, Fe, Zn, Se, and possibly Cu, Mn, Mo, and V, which are usually present as trace contaminants) are required. The commonly used CHO derivatives DXB11 and DG44 have an absolute requirement for proline that results from a genetic deficiency in the conversion of glutamic acid to glutamic γ-semialdehyde. Inorganic ions may function as catalysts or exert physiological effects. Glucose (5 to 20 mM) and glutamine (0.5 to 5 mM) are commonly used to provide the energy source for the cells. These are consumed at a rate proportional to the viable-cell concentration. Cultured mammalian cells do not use the complete tricarboxcylic acid (TCA) cycle, and the glucose-consumption rate is lower than the glutamine consumption rate. Consumption of glucose yields lactate and consumption of glutamine yields ammonia as the metabolic byproduct. Other nutrients are consumed at a rate proportional to the rate of increase in cell density. Yield-based uptake rates for amino acids by CHO DG 44 cells range from 0.1 mM tryptophan/10^9 cells to 0.8 mM arginine/10^9 cells at a specific growth rate (the rate of growth divided by the cell density) of 0.25 to 0.30 $days^{-1}$ (D. Gray, unpub. observ.), reflecting the relative incorporation frequency of the individual amino acids into general protein. CHO cells, like most mammalian cells, produce alanine as a result of glutaminolysis. Accumulation of ammonia, as a result of glutamine degradation in the medium and consumption by cells, may become inhibitory to cell growth and ultimately limit the density of the culture (Butler and Spier, 1984).

In addition, physiological parameters such as pH, osmolality, and redox potential must be kept within acceptable limits. The use of serum is often advocated and has been regarded as a source of important components such as hormones, lipids, and growth factors. These serum-borne ingredients provide some consumable nutritional components as well as mitogens or growth factors that overall are not consumed by the cells (Klinman and McKearn, 1981).

A further level of complication in mammalian-cell medium design occurs through the need to maintain balanced relationships between nutrients while maintaining the osmolality of the medium within the physiological constraints (290 to 380 mOsmol/kg).

Recent medium development has focused on reducing the need for high levels of serum (Jayme, 1991). Fetal bovine serum (FBS) is the most frequently used version of serum and can be obtained from HyClone (see *SUPPLIERS APPENDIX*). FBS costs ~$500 to $600 per liter and is a factor contributing to the high cost of producing commercial clinical lots of protein. There may be batch-to-batch variation in serum which necessitates screening by small T-flask or shake-flask growth studies with the chosen mammalian cell line. Serum contains ~35 mg/ml of protein, of which ~60% is albumin. Thus if 10% serum were added to medium, the level of initial exogenous protein in the media would be 3 to 5 mg/ml.

Growth of cells in low serum (<0.5% v/v) has been accomplished through step-wise adaptation of cells to growth in medium with low or zero serum. The important functions of serum are replaced by the addition of the lipid precursors choline, inositol, and ethanolamine, the polyamine putrescine, and recombinant insulin (Nucellin-Zn from Eli Lilly) at 1 to 5 mg/liter (Jenkins, 1990) along with $FeCl_3$/citrate (Keenan and Clynes, 1996). A number of serum-free media have been developed and are now commercially available from, e.g., BioWhittaker. Another approach to implementing protein-free media has been the genetic modification of CHO cells through incorporation of the heterologous genes coding for transferrin and IGF-1 (Pak et al., 1996). As a result of cellular expression of the IGF-1 and transferrin, the cell is then able to grow, without addition of exogenous proteins, in a modified DMEM/F12 medium supplemented with sodium selenite.

A major concern in producing recombinant protein in protein-free medium is the potential for proteolysis and glycosidase activity. Such activity results in processing of the polypeptide chain and degradation of the carbohydrate moiety (Gramer and Goochee, 1993; Teige et al., 1994). Serum contains endogenous protease inhibitors—e.g., α_2-macroglubulin (Barrett, 1981)—and for this reason may be a valuable reagent in its own right in cell culture media

when the produced protein is susceptible to proteolysis. Sialidases retain significant activity at pH 7.0 and are active in cell culture supernatants. Caution should be taken to minimize cell lysis during the culture process if the goal is to minimize desialylation of the expressed protein. This may be achieved by harvesting the supernatants during the exponential-growth phase prior to the beginning of the decreasing-viability phase, chilling it, and adding sialidase inhibitors such as 2,3-dehydro-2-deoxy-*N*-acetylneuramic acid to the supernatant (Gramer et al., 1995). In batch culture in serum-free medium, cell viability may decrease rapidly at the end of culture, thereby releasing intracellular proteases and glycosidases into the culture medium, which may in turn cause rapid proteolysis and carbohydrate degradation of the product.

Production of sialylated forms of glycoproteins is important in clinical applications to avoid rapid clearance by asialo- and asialogalactoprotein receptors in the liver. The genes for some of the key enzymes in the glycosylation pathway have been transfected into CHO cells (Lee et al., 1989) in attempts to tailor the glycoforms. A number of CHO-glycosylation mutants have been made available that exhibit a defect in one or more steps in the pathway (Stanley, 1989). Erythropoietin (EPO) expressed in the CHO mutant Lec 3.2.8.1. exhibited less heterogeneity than EPO produced in wild-type CHO cells (Stanley, 1991).

Studies with CHO-320 cells in glucose-limited chemostat culture (using RPMI-1640 medium) at a low dilution rate of 0.008 day^{-1} (i.e., volume of fresh medium added to the reactor per day divided by the reactor medium volume) showed a deviation of the specific growth rate from the dilution rate and an increase in specific cell-death rate (i.e., rate of cell death per liter per day divided by the number of viable cells per liter), suggesting that a minimum specific growth rate of 0.011 day^{-1} existed (Hayter et al., 1993). In the same study, the specific rate of γ interferon (γ-IFN) production increased with the specific growth rate (μ), indicating growth-related product production. The pattern of γ-IFN glycosylation was similar at all growth rates except the lowest, where an increase in nonglycosylated γ-IFN was observed. This was further addressed using batch culture (Leelavatcharamas et al., 1994), where it was concluded that the growth-associated production was a reflection of a cell cycle–related phenomenon whereby lower growth rate resulted in a prolongation of the cell-cycle time with an increase in the relative frequency of the G_1 cells in the culture population. It was suggested that the G_1 phase is the least productive phase in the cell cycle of CHO cells. Similarly S phase–specific synthesis of dihydrofolate reductase in nontransfected CHO-K1 cells was observed in which the maximum peak of DHFR activity coincided with the maximum rate of DNA synthesis in late S phase (Mariani et al., 1981). The expression of recombinant β-galactosidase by the CMV late promoter in CHO βG72-16 was shown to be S-phase related (Gu et al., 1993). It is likely that CHO cells will generally exhibit an S-phase dependency for expression of foreign genes using the typical promoter elements such as the CMV late promoter, and that a direct correlation of specific heterologous protein production rate with specific growth rate will be observed (Bock et al., 1993; Gu et al., 1996). As growth rate increases, the ratio of $S/(G_1 + G_2/M)$ increases (Hooker et al., 1995) and the frequency of cells in productive S phase in the culture increases.

A given glycoprotein produced in cell culture by mammalian cells will present a population of molecules exhibiting differences in glycosylation. The existence of families of structurally related yet distinct carbohydrate chains makes structural analysis of a distinct species complicated, since it is difficult to separate and purify the structural variants. Variation may result from incomplete occupancy of a directed site on the protein and from carbohydrate-structure variations imparted to the cells via cell culture–based conditions. Furthermore the action of glycosidases in the extracellular environment, particularly during batch culture, may degrade the carbohydrate structure of secreted glycoprotein, leading to a wider range of carbohydrate structures in the population. Changes in the oligosaccharides of the glycoprotein may lead to diminished biological efficacy in vivo—e.g., through increased clearance—or may enhance unwanted antigenicity of the molecule in a clinical application (Goochee et al., 1991).

Antibodies produced in CHO cells in serum-containing medium exhibited more galactosylation compared to antibodies produced by cells grown in serum-free medium (Lifely et al., 1995). The culture-medium glucose concentration affected the degree of glycosylation of γ-IFN produced in CHO cells in continuous culture (Hayter et al., 1992). Lipid supplements, with or without lipid carriers such as lipoprotein, appeared to increase N-glycosylation-site occupancy in γ-IFN (Jenkins et al.,

Table 5.9.3 Estimated Quantity of Foreign Protein Produced by a Given Production System[a]

System	Vol. of medium in system	Total cells in system (vol. × conc.)	Total product in system[b]
Batch T-flask (T-175):			
175 cm^2 attached growth in DMEM/F12/10% serum[c]	75 ml	1.8×10^7	0.5-1 mg
Batch shake flask (250 ml), 0.5-1% serum[c]	100 ml	2×10^8	2-4 mg
Batch 5-liter spinner flask[c]	1000 ml	1×10^9	10-20 mg
Batch 5-liter spinner flask fitted with O_2 frit sparger[c]	1000 ml	2×10^9	20-40 mg
Batch 15-liter bioreactor with pH and O_2 control[c]	10 liters	2×10^{10}	200-400 mg
Continuous-mode 15-liter perfusion reactor with pH and O_2 control and cell retention	5-20 liter perfusion per day	1×10^{11}	100-400 mg per day

[a]Quantities assume a specific productivity of 1 to 4 mg/10^9 viable cells per day.

[b]Production of recombinant intracellular protein may achieve ~0.1% of total cell protein.

[c]Batch culture would be performed for 4 days after inoculation of 1×10^5 viable cells/ml.

1994). In perfusion culture of BHK-21 cells producing a recombinant interleukin 2 (IL-2) mutant, nutrient limitations (i.e., glucose, amino acids, and dissolved O_2) led to short-term changes in the general level of glycosylation, but the specific type of sugar incorporated was largely unchanged (Gawlitzek et al., 1995).

Direct intervention in cell physiological processes has an impact on glycosylation. A decreased protein-synthesis rate following addition of cyclohexamide improved the glycosylation-site occupancy of recombinant prolactin produced by C127 cells (Shelikoff et al., 1994). Low concentrations of dithiothreitol, which prevent cotranslational disulfide-bond formation in the ER, led to complete glycosylation of tPA at a tripeptide sequon that normally exhibited variable occupancy (Allen et al., 1995). This suggests that refolding may in itself limit the accessibility of glycosylation sites.

Sodium butyrate is sometimes used to improve protein synthesis, but it can change glycosylation by inducing the GlcNAc-transferase involved in O-glycosylation (Datti and Dennis, 1993) and by increasing sialyltransferase activity in recombinant CHO cells (Chotigeat et al., 1994; Gebert and Gray, 1995).

SCALE OF OPERATION

Batch growth in shake flasks or spinner flasks may provide titers in stable producer cell lines of 10 to 50 mg/liter, thus yielding up to 30 to 150 mg of crude secreted product which may then be purified from the culture supernatant. Following purification, the researcher may recover ~10% to 30% of the protein. Thus, small laboratory-scale cell culture may provide 3 to 50 mg of pure protein (>90% purity). Larger quantities of material require use of bioreactors in which pH, dissolved O_2, temperature, and stirring may be controlled. Should the user wish to control the cell growth rate during the production of the product, a continuous reactor may be used at a fixed dilution rate. In cases where high cell density is desired, the mode of operation would be continuous medium perfusion with cell retention in the bioreactor. Tables 5.9.3 and 5.9.4 provide some guidance for the likely scale requirements for a given objective.

The bioreactor environment may influence the product quality. Mild hypoxia may decrease the specific activity of sialyl transferase and hence the sialylation of the oligosaccharide chain, as was observed with recombinant human follicle-stimulating hormone produced in CHO cells at 10% dissolved oxygen saturation (Chotigeat et al., 1994). It appears that pH values within the range 6.9 to 8.2 have no dramatic effect on glycosylation, but clearly optimal growth would require control of pH within a narrower range, typically 7.0 to 7.4.

Metabolic byproducts may be the limiting factors in achieving high cell density in perfu-

Table 5.9.4 Typical Protein Requirements for Various Analyses

Assay	Quantity of protein required	Comments
ELISA	<5 μg	Identification of activity possible with crude extract
Amino acid analysis and sequencing (including development of method)	20 μg	Pure protein required; thus starting requirement would be >100 μg in order to account for purification losses
Carbohydrate analysis	20 μg	Pure protein required
Extinction-coefficient determination	500 μg	Pure protein required; thus starting requirement would be 3-5 mg
Biological testing in animal-based model	50 mg	Must obtain suitable purity of product to prevent collateral effects on physiology due to contaminants
Human clinical trials	1-100 g	>95% purity; requires extensive testing to ensure safety

sion culture. Increases in ammonium ion concentration to 10 mM in the culture medium resulted in substantial growth inhibition in hybridoma cells (McQueen and Bailey, 1990) and appeared to inhibit growth in most cell lines. The likely cause of this is the transport of ammonium ion by membrane-bound ion pumps and ultimate acidification of the cytoplasm (Martinelle et al., 1996). Lower levels of ammonia (~2 mM) may exert influences on glycosylation (Andersen et al., 1994). One approach to eliminating this potential inhibition of cell growth is through adaptation to a nonammoniagenic medium. This medium would contain glutamate or 2-oxoglutarate in place of glutamine (Hassel and Butler, 1990). Lactate generated from glucose can also reach growth inhibitory levels at >15 mM. Optimization of medium and perfusion rate may enable reduction in the levels of lactate and ammonia, allowing higher cell density. Lowering the glucose level may reduce the yield of lactate, but concomitant limitation of glycosylation may occur. CO_2 may accumulate in high-density cultures, thereby exerting an inhibitory effect on the culture (Gray et al., 1996). Limitations that are due to CO_2 accumulation may be overcome by choosing an optimal sparging strategy.

Finally mammalian cells are weaker and larger than bacterial cells and limits to the degree of agitation are important to prevent cell damage and death. Where agitation is used in reactors or in sample handling, cell damage at the gas-liquid interface as well as bubble damage can be reduced by adding low levels—<0.1% (w/v)—of the surfactant pluronic polyol (Murhammer and Goochee, 1990).

SUMMARY

Both yeast and *E. coli* heterologous protein expression are very well defined. The components of these expression systems are easily obtained through many commercial vendors. Use of these systems enables the researcher to obtain large amounts of pure material in a short period of time. In contrast, mammalian expression systems are not comprehensively defined or understood and require a significant investment of time before heterologous protein is obtained. At the present time the mammalian expression systems are not available as turnkey, catalog-based systems. The user must define the components for a particular application. Although this complexity exists with the mammalian cell–based systems, they are uniquely important for the production of glycoproteins that possess oligosaccharides close to or identical to those of human origin. Proteins secreted by mammalian cells possess correct conformations and exhibit full biological activity. The ability to transfer foreign DNA into these cells enables a greater understanding of the mammalian cell transcriptional, translational, and post-translational machinery. The ability to incorporate foreign gene sequences into a mammalian-cell chromosome and the ability to grow mammalian cells in suspension in large-scale bioreactors has enabled the production of large quantities of pure clinically important proteins.

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