Preface

The essential and determining feature of an industrial bioprocess is the culturing of cells that yield a desired product. Mammalian, microbial, and plant cells are traditionally used for the manufacture of products derived directly or semisynthetically from cellular metabolites. These cells are increasingly used as the cellular machinery to express recombinant proteins of considerable economic and therapeutic value. The choice of cell culture type determines the degree of success in obtaining a clinically useful product, as well as in achieving an economical process, by facilitating acceptable yield and purity. Each of the major classes of industrially relevant cultures is manipulated by a variety of means, selected for desired phenotypes, and is exploited either in bioreactors or in the field by functionally similar approaches.

The knowledge of how best to achieve this utility has its roots in empirical learning that reaches back many thousands of years. Much later, Pasteur, Koch, and others dramatically advanced our knowledge of the underlying cellular nature of bioprocesses during classical studies in the 19th century using modern scientific methods. Following World War II, the advent of modern industrial production methods, inspired by the discovery and isolation of penicillin, brought the first boom in natural product biotechnology. More recently, the dramatic acceleration in identifying protein biopharmaceutical candidates, as well as the current rebirth in natural product discovery, have been driven by molecular genetics. Likewise, plant cell culture and engineered crops have already impacted agriculture and are poised to revolutionize biotechnology.

The progression of transgenic animal and plant methodologies from laboratory to industrial scale production has resulted in the most recent, and perhaps most dramatic, step in using cells to make products. Supporting the production of novel therapeutics in mammalian, microbial, and plant cells is an impressive array of new methodologies from the fields of molecular genetics, proteomics, genomics, analytical biochemistry, and screening. For an industrial bioprocess, manipulation and propagation of cells in order to elicit expression of a product is followed by the recovery, analysis, and identification of these products. The methodology for successfully developing a commercial process is functionally similar across the spectrum of cell types.

Handbook of Industrial Cell Culture: Mammalian, Microbial, and Plant Cells attempts to link these common approaches, while also delineating those specific aspects of cell types, to give the reader not only an overview of the best current practices, but also of today's evolving technologies, with examples of both their practical applications and their future potential. Many scientists currently in the field find their careers transitioning across work with mammalian, microbial, and plant bioprocesses; thus they are very much in need of a book linking these disciplines in a single format. Moreover, the next generation of scientists and engineers will interface across these disciplines and likely see even more dramatic enhancements in technology. Our hope is that this Handbook will prove especially useful not only to those involved in biotechnology as a broad discipline, but also assist experienced practitioners in perfecting the special art of industrial cell culture.

Victor A. Vinci
Sarad R. Parekh

2

Genetic Approaches to Recombinant Protein Production in Mammalian Cells

Peter P. Mueller, Dagmar Wirth, Jacqueline Unsinger, and Hansjörg Hauser

1. Control of Gene Expression

Genetically engineered mammalian cells play an essential role in many processes, from basic research to high-throughput screening and pharmaceutical protein production. In basic research, mammalian cells serve to study gene function and mechanisms of regulation. Important health-related applications include drug screening and production of secreted, pharmaceutically active proteins. The reason that mammalian cells are preferred is the close relationship to cells and their products in the human body. In particular, mammalian cells have the unique capability to authentically process, fold, and modify secreted human proteins. The resulting products are free of microbial contaminants, thereby minimizing the risk of immunogenic and inflammatory responses, respectively. In addition, human-like modifications extend the in vivo lifetime of therapeutic proteins. This translates into therapeutic products that are safe and highly active.

However, compared to the alternative cell-culture systems, protein production in mammalian cells is time-consuming and expensive. Multiple factors affect recombinant protein production in mammalian cells, such as the specific cell line used and the expression vector, chromosomal integration site, and copy number of the integrated recombinant gene, selection procedures, cell-culture conditions, and medium employed *(1)*.

A standard recombinant protein production strategy is to first choose a suitable producer-cell line. Then, the DNA carrying the gene of interest and a selection marker gene are transfected into the host cells. Only a small minority of the transfected cells will integrate the recombinant DNA into the genome. Cells that express the selection marker grow and form clones under appropriate conditions, whereas untransfected cells are eliminated. However, there is little correlation between selection-marker gene expression and the expression level of the gene of interest. The productivity of newly isolated clones is unpredictable, variable, and usually modest. Since the optimal producer clones are generally not those with the highest initial productivity, a large number of clones must be screened and characterized further. Limitations may occur at various levels within a producer-cell clone, including transcriptional silencing, inefficient or aberrant mRNA processing, instability of the recombinant mRNA, low transla-

From: *Handbook of Industrial Cell Culture: Mammalian, Microbial, and Plant Cells*
Edited by: V. A. Vinci and S. R. Parekh

tional efficiency, and bottlenecks in post-translational modifications and in secretion. In addition, mammalian cells are fragile, they grow slowly, and require complex media and sophisticated fermentation setups for the production process. Because of the limited maximal cell densities achieved, the overall production is one or more orders of magnitude below values achieved with bacterial, fungal, or insect cell systems. For these reasons, sophisticated vector design and elaborate producer-cell adaptation and optimization procedures are used to improve process productivity.

Interestingly, in their native environment highly specialized cells represent the most efficient mammalian expression systems known in the body, such as ß-globin synthesis in erythroblasts or immunoglobulin secretion by B-cells. Understanding the principles of gene expression in such cells could lead to recombinant cell lines with superior production properties.

1.1. Vector Design

Generally, mammalian expression vectors are circular shuttle plasmid vectors (Fig. 1). Such plasmids usually carry a mammalian gene-expression cassette and an optional mammalian selection-marker gene. Alternatively, a mammalian selection marker can be provided on a second plasmid that is co-transfected with the expression construct. Since standard transfection protocols result in the integration of multiple copies of the transfected DNA into the mammalian genome, the frequent co-integration of the expression construct and at least one copy of a drug-resistant gene results in the selection of mostly producer cells in the presence of the drug. To facilitate vector DNA preparation, an origin of replication and a bacterial selection marker gene allow for the propagation and selection of these plasmids in *E. coli*.

In most cases, the expression of the gene of interest is driven by strong constitutive viral or cellular promoters, such as the cytomegalovirus (CMV) promoter or the translation elongation factor EF2α gene promoter. Mammalian promoters are generally extremely complex, but contain two basic parts, enhancer sequences and a minimal promoter sequence (Fig. 1). Enhancers often contain repeated sequences that are binding sites for transcriptional activators, whose position relative to the transcription start site can be surprisingly variable, and can even function downstream of the transcribed region. The minimal promoter sequence binds factors of the basic RNA polymerase II-dependent transcriptional machinery and determines the transcriptional start site. In practice, natural promoters are much more complex, and the interactions and contributions of the various transcriptional elements have rarely been investigated in detail. However, the composite nature of mammalian promoters allows construction or adaptation for special purposes. In particular, for regulated gene expression, enhancer elements of the CMV promoter can be replaced by binding sites for recombinant or regulated transcription activators such as binding sites for the tetracycline-regulated transactivator. In addition, cell-specific enhancers can even be added downstream of the transcription unit to increase expression in the cell line of interest. Immediately downstream of the promoter, multiple unique restriction endonuclease recognition sequences (multiple cloning site) allow the insertion of the reading frame of interest.

Most mammalian genes contain introns. For recombinant gene expression, the much shorter minimal protein coding sequences that are derived from reverse transcription of

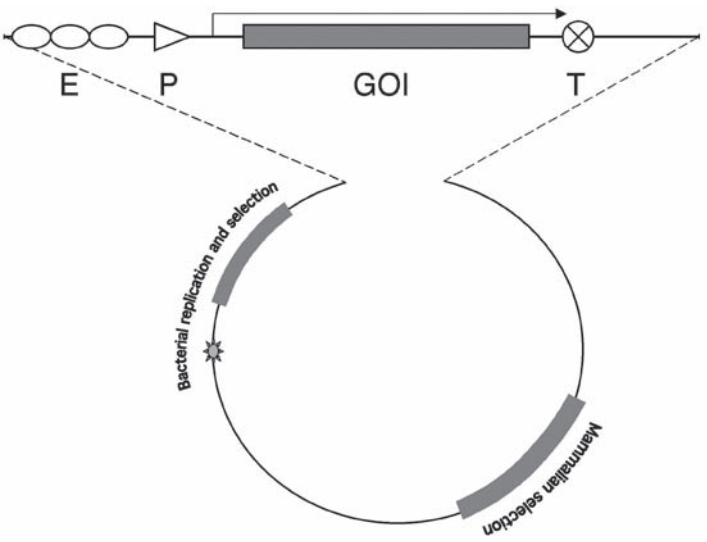

Fig. 1. Typical components of a basic expression vector. An enhancer (E) consisting of multiple transcriptional activator binding sites is a major determinant of the transcriptional activity. The minimal promoter element (P) frequently contains a conserved sequence element (TATA box) that constitutes a binding site for the RNA polymerase II transcription factor TBP and determines the transcription initiation site (arrow). Transcription of the reading frame of the gene of interest (GOI) is terminated by an mRNA precursor cleavage site and poly(A) addition signal (T) that defines the 3' end of the mRNA. Optionally, the vector may carry a second transcription unit encoding a mammalian selection gene. A bacterial origin of replication and an antibiotic resistance selection gene facilitate DNA manipulations, cloning, and plasmid DNA preparation in *E. coli*.

mature intronless mRNA (cDNA) are often used. However, introns and splice sites are also recognition sites for proteins that facilitate the mRNA export from the nucleus. Therefore, some expression constructs that contain a natural or artificial intron are more efficiently expressed than an intronless version of the same gene. Further improvement of gene expression can sometimes be achieved by replacing unfavorable secondary RNA structures and upstream initiation codons in the 5' noncoding region with a favorable unstructured 5' mRNA with an optimal consensus initiation sequence *(2)*, or by removing mRNA destabilizing sequences downstream of the protein coding region that are present in many relevant cytokine mRNAs. To avoid the generation of unstable transcripts, the protein-coding region must be followed by a cleavage and poly(A) tail addition site. This appears to be a somewhat less critical choice than promoter selection, and there are various efficient poly(A) addition sites available that are currently used.

1.2. Regulated Gene Expression

Regulated promoters can be used to restrict recombinant gene expression of toxic or growth-inhibitory products to the final production phase, in which further cell growth is no longer essential. Many such regulatory systems are available that respond to spe-

cific medium additives or to growth conditions. One major goal is to achieve efficient expression under inducing conditions while keeping basal expression low in the noninduced state. Presently, the most popular system for this purpose consists of two elements, a chimeric transactivator protein and a DNA construct containing an inducible promoter with multiple transactivator binding sites *(3)*. The transactivator tTA comprises the DNA-binding domain of the prokaryotic tetracycline repressor protein fused to a eukaryotic transcriptional-activating domain. In the original construct, DNA-binding of the tTA transactivator is prevented in the presence of tetracycline. When tetracycline is omitted, the tTA protein binds to the cognate promoter and efficiently activates transcription. The system has been developed to such a high degree of sophistication that it is the method of choice for regulated expression. In some cell lines, very high transcription rates have been achieved, as well as ranges of expression spanning five orders of magnitude *(3)*. In addition to the tetracycline-repressed transactivator, an inverse system that is tetracycline-inducible has been developed *(4)*. The level of the transactivator protein present in the cell plays a major role in the transcriptional activity of the tetracycline-dependent promoter. A number of such cell lines expressing tetracycline-regulated transactivators as well as the tTA-dependent promoter system are commercially available (Tet-On™/Tet-Off™; Clontech).

Another development of the tTA system are autoregulatory expression systems in which both, the gene of interest and the transactivator are expressed from the tTA-dependent promoter. Low basal levels of transactivator gene expression allow self-amplified activation upon withdrawal of tetracycline. In addition, tTA overexpression can negatively affect the cell growth rate or even reduce cell viability. An advantage of autoregulation is that tTA-expression and its potentially toxic side effects are restricted to the time of induction. Furthermore, the autoregulatory cassettes can be expressed in a tissue-independent manner *(5)*. Because of the necessary minimal basal expression level in the repressed state, autoregulatory expression units cannot be applied for expression of highly toxic proteins, which are lethal even if expressed in minimal amounts. Autoregulatory expression cassettes based on modified bidirectional tTA-dependent promoters have been established *(6–8)*.

A related streptogramin-regulated system is available, which can be used as an alternative to the tetracycline system or in tandem with the same cells to regulate the expression of two different products independently *(9)*. Another type of regulated expression system that is widely used is steroid hormone-receptor-responsive transactivators. Hormone-dependent transcription activators have been constructed by fusing hormone-binding domains of steroid receptors to DNA-binding domains of unrelated heterologous proteins *(10)*. DNA-binding domains from the yeast *Saccharomyces cerevisiae* Gal4 protein have been used in combination with the hormone-binding domain of the mammalian estrogen receptor, which can be induced by estradiol *(11)*. Analogously, a progesterone-receptor fusion protein that can be activated by RU 486 at concentrations much lower than those required for antiprogesterone activity *(12,13)* and a system based on the heterodimeric insect ecdyson receptor can be induced by the synthetic ecdysteroid compound known as ponasterone A *(14)*. In the absence of hormone, the receptors form an inactive complex with heat-shock proteins. Ligand addition induces DNA-binding and transcriptional activation of the target promoters.

2. Dominant Role of the Chromosomal State in Gene Expression

2.1. Influence of Position and Gene Copy Number on Number on Transcription Levels

Transcription is the first step of recombinant gene expression, and has remained a focal point of interest. Most current applications make use of cell lines with recombinant genes stably integrated into the genome of a host cell under the control of a strong cellular or viral promoter *(15)*. Some of these viral promoters, including the CMV promoter *(16)*, can be used in different cell lines for high-level protein expression, but their transcriptional activity varies depending on the cellular levels of the relevant transcription factors, on the copy number of the integrated DNA, and on the integration site.

DNA in the cell nucleus is organized in chromatin that determines the transcriptional capacity of a particular chromosomal region to a large degree. Chromatin consists of DNA that is wrapped around histones, forming the so-called nucleosomes. Chromatin can condense into higher-order structures, resulting in tightly packed DNA-histone complexes that prevent access to transcription factors, producing inactive genes. The accessibility of chromatin to transcription factors is determined to a large degree by its acetylation status. Histone acetylation leads to a net increase in negative charges and is believed to loosen the histone-DNA interactions, thereby facilitating access to activating transcription factors. The acetylation status is determined by the antagonistic activities of histone acetyltransferases (acetylases) and histone deacetylases. Sequence-specific DNA-binding transcriptional activating proteins frequently recruit histone acetylases, whereas transcriptional repressors and silencers recruit histone deacetylases. The effects of these enzymes can be localized to a few nucleosomes, and can be specific for a given promoter. However, the acetylation status can spread from so-called locus control regions (LCR) over an entire chromatin domain and can affect multiple genes simultaneously. Further spreading is believed to be prevented by specialized DNA regions, such as insulator sequences *(17)* or AT-rich nuclear scaffold/matrix attachment regions, (S/MAR) elements *(18)* (*compare* Table 1).

Several strategies to maximize gene expression at the transcriptional level aim to increase histone acetylation. Expression vectors have been constructed that carry S/MAR elements to specifically protect the insert from negative influences of the surrounding chromatin at the integration site *(18)*. Alternatively, cell-culture medium additions of deacetylase inhibitors such as butyrate can increase recombinant gene expression by enhancing the overall level of histone acetylation *(24)*.

Aside from the chromosomal integration site, the copy number of the inserted gene can have a significant influence on the expression characteristics. On average, higher gene copy numbers correlate with higher expression levels. However, when individual clones are compared, this correlation breaks down, because of the drastic differences in the expression level of individual clones. Also, there appear to be cellular mechanisms that recognize repeated sequences and preferentially inactivate them transcriptionally in the absence of selection pressure, resulting in a gradual decrease in specific productivity. Thus, in some applications, stable expression from single or low gene copy numbers is preferred. Alternatively, recombinant protein overexpression in the most widely used Chinese Hamster Ovary cells (CHO) is achieved by gene amplification. To obtain such cell lines, an amplification marker—usually the dihydrofolate reductase gene

Table 1
DNA Elements That Can Affect Promoter Activity

Epigenetic element	Description	Reference
Enhancer	Increases transcription of nearby promoters	*(19)*
Silencer	Reduces transcription of nearby promoters	*(20)*
Scaffold/matrix attached region	Anchoring of DNA to the nuclear scaffold; structural boundaries of chromatin	*(18,21)*
Insulator	Boundary element, blocks enhancer and silencer action	*(17, 22)*
Locus control region (LCR)	Dominant activating sequence that confers position and copy number-dependent expression on a linked gene	*(23)*

(DHFR)—is cotransfected together with the gene of interest. Resistant cell clones that first have low expression levels of both the gene of interest and DHFR are successively selected for increasing resistance to the amplification drug (methotrexate). This is often caused by an increase in the copy number (amplification) of a chromosomal domain containing the amplification marker gene and the gene of interest *(25,26)*. The expression level of both heterologous genes increases with the domain copy number present in the genome. This procedure to isolate high copy number clones is extremely time-consuming. The initial expression level is not meaningful, and amplifiable clones cannot be identified in any other way. Therefore, a large number of clones must be handled initially, and the entire procedure to obtain a final producer strain may involve a period of several months to years. Because of the unstable expression characteristics, the cells must be cultivated under constant selection pressure. However, the presence of toxic substances in the final production process is generally omitted because of difficulties with the disposal of large volumes of toxic waste. Therefore, gene amplification using methotrexate is preferentially used in limited batch processes. Selection marker genes such as glutamine synthetase are used for continuous production processes by using nontoxic glutamine-free selection medium instead of methionine sulphoximine-containing medium. Depending on the particular product, final expression levels in CHO cells in the range of 10–100 pg/cell/d are generally considered satisfactory, although higher titers have been reported *(27)*.

A major drawback of isolating stable, inducible, or constitutive high-level producer-cell lines is usually a time-consuming selection procedure. In order to facilitate isolation of high-producing cell clones with desirable characteristics, a number of alternative techniques have been developed.

2.2. Controlled and Targeted Chromosomal Integration Expression Strategies

Although producer cells with multiple copies of the transgene often yield large amounts of the protein, the genetic instability of these multimeric cassettes requires continuous growth in the presence of the respective selection drug. Selected cell clones carrying a single copy of the transgene can overcome the problem of instability without a loss in productivity. However, high screening efforts usually must be made in order

to isolate appropriate clones. The novel strategies discussed in the following section provide a means to overcome this problem.

Generally, transgenes expressed from ectopic chromosomal sites are subjected to position-dependent expression phenomena. Such epigenetic features usually arise through local modifications by chromatin structure that generally result from interactions between the transgene and chromatin at the site of integration. In some circumstances, this process can be bidirectional, so that the transgene changes expression from the endogenous gene at the site of integration. Numerous experiments using transgenic animals have confirmed the complexity of this epigenetic influence on gene expression, and have shown that some genomic sites develop very complex interactions and others are essentially neutral, allowing a transgene to be expressed according to patterns seen at the natural chromosomal locus. In recent years, a number of chromosomal elements have been identified that are capable of influencing the expression of a transduced gene (Table 1). However, currently no efficient protocols exist to use these elements for efficient protection of a transduced gene from influences mediated by the nature of the integration site.

Nevertheless, many examples from practical applications show that single-copy integration of a transgene can lead to high and stable expression. This proves that favorable chromosomal sites exist in the genome of currently used production cell lines. However, to identify such high-expressing cell clones, a substantial effort is required. The major recurring task for every gene of interest is the time-consuming screening for appropriate levels of expression and stability followed by validation procedures.

Consequently, in recent years, efforts have been made in order to reuse one of the rare sites on the chromosome that allow high and stable gene expression from a single integrated gene copy. Classically, the specific targeting of a transgene to a predetermined chromosomal site can be achieved by homologous recombination. However, this method requires a detailed characterization of the bordering fragments and long stretches (~5 kb) of flanking DNA must be available to construct the recombination vector. In addition, the frequency of recombination also varies with the accessibility of the integration site, and appropriate selection strategies are required. Although this technique is routinely applied in murine ES cells for the establishment of transgenic mice, the method is hardly feasible for transformed cell lines used for biotechnological purposes. This is because in the latter cells, homologous recombination is masked by the more frequently occurring illegitimate recombination events that result in a random integration of the respective transgene *(28)*.

New perspectives have been reached by application of the sequence-specific recombinases Cre or Flp in mammalian cell lines *(29,30)*. These enzymes are derived from bacteriophage P1 and yeast, respectively, and mediate site-specific recombination between short recombination targets, so-called loxP (for Cre) and FRT sites (for Flp). These recombination targets consist of two inverted 13-basepair (bp) repeats flanking an 8-bp spacer element. In the FRT sites, a third inverted repeat is present. The recombinases bind to the inverted repeat elements and perform cleavage and religation of the spacer region. Elements flanked by two correspondingly orientated loxP or FRT sites are efficiently excised by the respective recombinase (Fig. 2A). Generally, these enzymes are also capable of catalyzing the backward reaction, which corresponds to a site-specific integration into a loxP or FRT tagged chromosomal

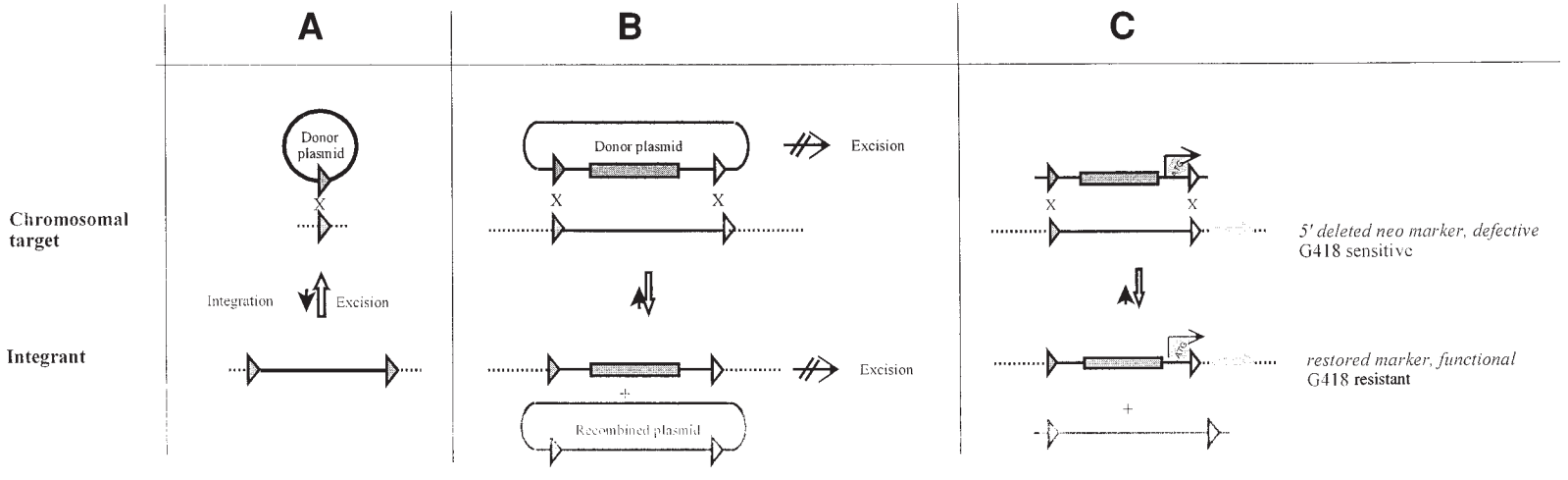

Fig. 2. Strategies for targeted integration. (**A**) Principle of Flp- or Cre-mediated integration. A donor plasmid tagged with a recombinase recognition target sequence can be specifically integrated into a correspondingly tagged chromosomal target site. However, the backward reaction is much more efficient. (**B**) Preventing excision by use of non-interacting recombination sites. A chromosomal locus is tagged with an expression cassette flanked by a set of non-interacting FRT sites (as symbolized by the black-and-white triangle). Transfection of a plasmid with corresponding FRT sites in presence of Flp recombinase results in the exchange of the intervening sequence. Excision is circumvented *(35,37)*. (**C**) Advanced targeted integration strategy. The efficiency can be further improved by combining the double FRT principle as shown in (**B**), with the recombination-mediated activation of a selection marker (*see* refs. *39,41*).

integration site. However, in this simple design, this method has not found a broad application. This is because the excision reaction is much more favored over specific integration both for thermodynamic and kinetic reasons: as soon as the integrated state is formed, it will be readily excised again in the presence of residual amounts of the recombinase. As a result, the intermediate integrated state cannot be fixed. Certain strategies restrict the action of the recombinase, either by expressing it in a timely restricted fashion *(31,32)* and/or by using (hormone-) activatable fusions *(33,34)*, yet could not significantly improve this application.

To overcome these limitations, a sophisticated version of this site-specific recombination strategy has been developed. This advanced strategy is based on sets of two heterospecific recombination target sites (spacer mutants). These target sites can recombine efficiently with homologous counterparts, but do not recombine heterospecifically. Such mutants have been developed for the Flp/FRT system *(35)*. Corresponding mutants for loxP have been developed, yet have not been shown to provide similar specificity *(36)*. Thus, an expression cassette flanked with a set of heterospecific mutants cannot be excised, and is stable even in the presence of the recombinase (Fig. 2B). However, such a cassette can be precisely exchanged for a novel cassette flanked with corresponding recombination target sites. Through application of a negative selection strategy (e.g., thymidine kinase) to eliminate cells which have not undergone recombination, cells with successful targeted exchange can be isolated *(37,38)*.

The efficiency of this approach can be further improved by combining the heterospecific FRT sites with a highly effective selection procedure for correct cassette replacement *(39)*. Thereby, the efficiency of targeted exchange is 90–100%, which reduces screening to a minimum. This strategy is based on an initiation codon-deficient drug-resistance marker, which can only be complemented by site-specific in-frame integration of the initiator codon (Fig. 2C). Only cells that have recombined precisely become drug-resistant. Most importantly, this technique has shown that the prediction of expression levels of a targeted transgene is feasible as long as the basic structure of the expression cassette is maintained *(40,41)*.

In summary, these advanced recombination-based techniques should now allow for a more rapid production of a new product. Integration sites with the desired expression properties are first screened using a reporter cassette flanked by heterospecific recombination target sites. Selected integration sites of choice are then efficiently targeted with the expression cassette of choice through a standardized process. The cultivation procedures developed for one such cell line could be applied to diverse products. Since all parameters except for the product gene are conserved, this would also greatly facilitate the approval of novel producer-cell lines by the regulatory authorities.

3. Co-Expression of Multiple Genes

Current applications in mammalian cell culture increasingly require the defined co-expression of different genes *(42)*. Four typical applications are described here: (i) Co-expression of a selectable marker together with the protein of interest, which is routinely used to establish stable cell lines. For industrial applications, overexpression of a recombinant protein in Chinese Hamster Ovary (CHO) cells is achieved by gene amplification (*see* **Subheading 2.1.**). (ii) Defined but unequal expression. Some applications require that the protein of interest is expressed at a much higher level than the

selectable marker. If a defined relationship of co-expression of both genes can be adjusted, cells that are resistant to the respective selective drug will produce the protein of interest at levels which cannot be below a critical threshold required for survival in the presence of the respective drug concentrations. (iii) Equivalent expression: expression of genes at a one-to-one ratio. Subunits of heteromultimeric protein complexes need to be synthesized in equal amounts. Examples are cytokine receptors, antibodies, or other di- or trimeric proteins. An additional application involves the co-expression at similar amounts of several enzymes that form a metabolic pathway. (iv) Quantitation of a protein for which no satisfactory assay method is available. If a strict coupling of expression of a reporter gene and the gene of interest is achieved [compare (ii)], a calibration for reporter and product level can indirectly determine the quantity of the protein of interest.

In nature, multiple levels of regulatory mechanisms ensure stoichiometric expression of genes in mammalian cells. Transcription, post-transcriptional processing, mRNA transport, stability, and translational efficiency adjust the correct levels of the synthesized proteins. A simulation of these events for genetic manipulation of cells would imply coordinated engineering efforts of all crucial steps. With the current techniques, this type of co-expression is nearly impossible to achieve. The currently applied methods for co-expression are summarized in Fig. 3.

3.1. IRES-Mediated Initiation of Translation

Although reinitiation of ribosomes is generally inefficient, internal ribosomal entry sites (IRES)-mediated internal initiation can be as efficient, or in rare cases even more efficient than cap-dependent initiation. Therefore, IRES elements are the method of choice to co-express multiple reading frames *(43)*.

IRES have been identified in viral and cellular eukaryotic mRNAs. Most currently well defined IRES elements are present in the 5' untranslated regions (5' UTR) of mRNAs and range from about 200–1300 nucleotides.

The type of IRES element used and its efficiency are of major importance for applications in expression vectors. The currently used classification of IRES elements is based on the picornaviral IRES classification *(44,45)*, which relies on the position of the initiation codon relative to the IRES element and additional downstream sequence requirements. Type I IRES elements include the Polio virus IRES element *(46)* and all cellular IRES elements characterized thus far. Type I IRES elements can be located at a variable distance from the downstream reading frame, and can be as far as 50–100 nucleotides upstream of the initiation codon. Type II IRES elements are characterized by the strict position requirements for the initiation codon at the 3' boundary of the IRES *(47)*. Encephalomyocarditis virus (EMCV) *(48)* is regarded as a prototype for class II elements. In type III IRES elements, the initiation codon position requirements are similar to type II IRES. However, additional sequences that are important for IRES function are located in the downstream coding sequence. The prototype of type III IRES is represented by the Hepatitis C virus (HCV) IRES.

A highly efficient cellular type I IRES element has been identified that shows a higher relative activity in various cell lines than picornaviral IRES elements (49). Cellular elements were discovered much later than the viral IRES, and presumably for this reason they are currently not used in such a broad range of applications as the viral

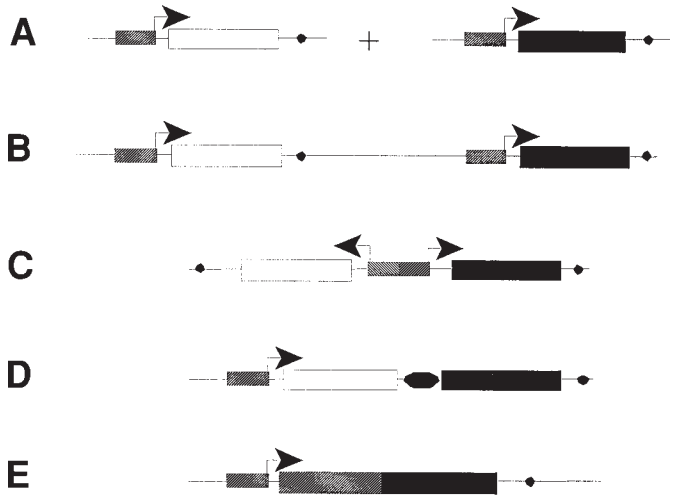

Fig. 3. Strategies for construction of polyvalent vectors (adapted from ref. *42*). Cassettes for the expression of two different genes (open and black boxes) under the control of unidirectional or bidirectional promoters (striped boxes) are shown. Arrows symbolize transcription starts, and filled circles depict polyadenylation sites. The filled oval symbolizes an IRES element. Vector sequences are drawn as black lines. A simple way to obtain co-expression of two proteins is to transfect cells with two independent constructs (**A**) or by introducing a single vector harboring two discrete expression cassettes (**B**). The first approach is often limited by the inefficiency and unpredictability of cotransfection. Another method relies on bidirectional promoters (**C**). Although this method is currently not in common use, it opens a new level of co-expression technology. The methods depicted from A to C suffer from the fact that even if the same promoter strength is given, the two transcripts may significantly differ because of variations in processing, half-life time, and translational efficiency of the mRNA. Di- and polycistronic mRNAs can be constructed by using internal ribosomal entry sites (IRES) that allow the co-expression from a single mRNA (**D**). In this way, variable expression ratios resulting from unpredictable transcription efficiencies of separate expression cassettes are circumvented. Finally, for special applications, fusion proteins provide the strictest coupling of two protein functions (**E**). However, for various reasons, this is often not possible.

ones. The use of various IRES elements facilitates the construction of multicistronic vectors with more than two reading frames by avoiding the potential for genetically unstable repeated insertion of identical IRES sequences.

Artificial IRES elements have been developed. A 9-nt segment of a cellular mRNA functions as an IRES, and when present in linked multiple copies, efficiently directs internal initiation *(50)*.

3.2. Polycistronic Vectors

Many commercially available dicistronic expression cassettes harbor a selectable marker or a reporter gene 3' of an IRES element and a multiple cloning site (MCS) for the insertion of the gene of interest. These cassettes are transcriptionally initiated by a promoter/enhancer for cap-dependent expression of the first cistron, followed by an

IRES element to permit translation of the second cistron and also provide a polyadenylation site at the 3' end. The insertion of genes into such vectors is straightforward but it is difficult to replace the second cistron by other genes needed.

Vectors or vector systems that allow the construction of di-, tri-, or multicistronic expression cassettes have been developed. Translation of mRNA that encodes the reading frames occurs upon cap-dependent initiation for the first cistron, and translation reinitiation of the second and all other downstream cistrons mediated by IRES elements. In most cases, IRES elements from Polio virus or EMCV are used *(51–57)*.

Compared to monocistronic mRNAs, dicistronic mRNAs are considerably longer and more complex. To achieve optimal expression, care must be taken to avoid the unintentional inclusion of special regulatory sequences such as polyadenylation signals or mRNA destabilizing elements. The expression level depends not only on the particular IRES element used, but also on both the sequence and on the order of the reading frames.

IRES-mediated translation efficiency is highly context-dependent, requiring experimental confirmation of the expression characteristics of each individual vector construct *(58)*. A systematic study of dicistronic vectors expressing two unrelated luciferase genes from Renilla and firefly, respectively, showed that the presence of the firefly luciferase in the first cistron has a surprisingly drastic inhibitory effect on internal initiation. This effect is independent of the promoter and IRES element used, and is observed in several cell lines *(59)*. Therefore the nature of the cistrons and their positioning is of key importance for their expression.

Overall, the efficiency of IRES elements can vary considerably, depending on the particular construct, on the experimental setup, or on the host cell used, and in some cases is even dependent on the physiological status of the cells. Despite the unquestionable advantages of multicistronic expression vectors, each individual construct must be tested, and if necessary, expression levels must be optimized to achieve satisfactory results.

3.3. Bidirectional Promoters

Coordinate expression of two transcription units can be obtained by using bidirectional promoters. Although for viral bidirectional promoters (e.g., SV40 and adenovirus) expression of the divergent transcription units is temporarily controlled in the course of infection mammalian bidirectional promoters allow simultaneous transcription in both directions. Most of the known mammalian bidirectional promoters are TATA box-deficient, and mediate low-level transcription of housekeeping genes. Generally, they are asymmetric, and one direction is preferentially transcribed.

The general potential of artificial bidirectional promoters for simultaneous expression of two genes has been recently shown for the artificial promoter P_{bi-1} *(60)*. This promoter is derived from a unidirectional tetracycline promoter *(3)*. The promoter is regulated by a tetracycline-dependent transactivator (*see* **Subheading 1.2.**).

The potential of the bidirectional promoter can be substantially extended if it is combined with polycistronic expression cassettes. Although simple cloning vectors are not yet available, the advantages of such a vector design are obvious: two coordinately transcribed mRNAs encoding several cistrons can be achieved. Thereby, certain problems or limitations arising from IRES-mediated translation of certain genes can be bypassed.

Applications of IRES elements and bidirectional promoters as outlined here are not restricted to DNA vectors. The expression cassettes described may be used to construct viral vectors and by this method, gene-transfer recombinant cells or organisms can be obtained with much higher efficiency.

4. Viral Vectors

One alternative to the transfection of DNA is the use of viral vector systems. Apart from an efficient transduction of different cell lines, viral systems offer high-level transient or stable gene expression, respectively.

A wide variety of viral vectors are used to deliver recombinant genes into mammalian cells, either in vitro (in established immortalized cell culture) for basic and applied research and particularly in vivo (in animals) and ex vivo (in primary cell cultures) for gene-therapy purposes. Viruses have developed highly efficient strategies to enter host cells and to alter the physiology of the host in order to achieve optimal conditions for viral gene expression.

In general, these viral properties can be used for the expression of a desired recombinant gene. For this purpose, certain viral sequences are replaced by the gene of interest. To allow packaging, the deleted viral proteins are offered in trans. Therefore, so-called packaging or helper cell lines have been constructed that express the missing viral proteins. The transfer of the recombinant viral vector into a helper-cell line results in the packaging of the vector into infectious viral particles. The particles are released from the cell, and can be used for infection. This strategy restricts the amplification of the virus to the packaging cells. Once the virus has entered the target cell, viral amplification is not possible because of the lack of viral proteins.

Since the virus species differ within their specific properties, the selection of the viral system depends on the experimental requirements. One of the major selection criteria is the requirement of transient or stable expression of the transgene.

4.1. Viral Vectors for Transient Expression

Viral vectors are used for transient gene expression in mammalian cells as an alternative to the cumbersome and time-consuming selection procedure of stable cell lines, because they are more efficient than nonviral transfection strategies for rapid and efficient but limited production of small quantities of recombinant product protein. Alphaviruses and adenoviruses, the most commonly used systems for transient protein production, will be discussed in the following section.

4.1.1. Alphaviruses

Alphavirus-derived vectors have been developed for protein production *(61)*, since they have a broad host range and can even efficiently infect nondividing cells. Semliki Forest virus (SFV), Sindbis virus (SIN), and several pathogenic encephalitis-producing viruses are all members of the alpha virus genus *(62)*, which replicates in a large number of animal hosts ranging from mosquitoes to avian and mammalian species *(63)*.

The typical alphavirus consists of an enveloped nucleocapsid containing a single-stranded, positive-polarity RNA genome of approx 12,000 nucleotides that is capped at the 5' end and polyadenylated at the 3' end. The RNA consists of two expression units with an internal promoter that mediates transcription of a subgenomic mRNA. The

viral structural genes encoded by the subgenomic transcription unit can be replaced by the gene of interest. The alphaviral genomic mRNA encodes its own replicase. The replicase mediates the replication of the plus-strand genome into full-length minus strands, which efficiently produce both new genomic and subgenomic RNAs that encode for viral proteins. Natural alphavirus gene expression and replication results in cell lysis caused by the avalanche-like amplification of the genomic mRNA. Replication of alphavirus-based systems is extremely efficient, leading to approx 10^5 new virions per cell. In this manner, the host's own translational machinery is used.

This replication efficiency has been utilized for the development of RNA- and DNA-based expression systems for the production of recombinant proteins in eukaryotic cells. *(61,62,64–68)*.

Expression vectors have been developed from SFV, SIN, and Venezuelan Equine Encephalitis virus (VEE). Since the alphaviral genome consists of a positive single-stranded RNA, the alphaviral RNA vector can be delivered directly either as naked RNA, as naked DNA, or as viral particles (Fig. 4). In all cases, high-level expression of the heterologous proteins is obtained. For virus production, the alphaviral recombinant RNA is cotransfected with a helper vector. High viral titers of approx 10^9–10^{10} per mL can be produced.

Since the large-scale production of RNA in vitro is expensive, a strategy where DNA as vector is used have been developed (Fig. 4c) *(69–71)*. In this system, the full-length recombinant alphaviral cDNA under control of an eukaryotic promoter is delivered directly to the cell by conventional DNA transfection. In the cell nucleus, the complete transferred unit is transcribed into RNA and transported to the cytoplasm. Translation of the RNA results in the production of the viral replicase, which initiates the replication of the entire molecule (*see* ref. *65*).

Alphavirus vectors have been used to express proteins for many different purposes, including large-scale production, protein characterization, and functional studies. In addition, alphavirus vectors can also be used for gene delivery in vivo with potential applications in vaccination and gene therapy *(72)*. One disadvantage is that for every production cycle of the gene of interest, DNA or viruses must be newly transferred.

4.1.2. Adenoviruses

Adenoviral transfer systems allow easy production and concentration to high viral titers (up to 10^{12} colony-forming units [CFU]/mL), a high level of transgene expression and a broad host range. Various cell types, including nondividing cells, can be infected. However, because of the lack of stable integration of the DNA, expression is only transient in proliferating cells, whereas in nonproliferating cells the genome persists as an episome and continues to express for a longer period of time.

Adenoviruses are nonenveloped viruses with a double-stranded DNA genome of 36 kb. Viral replication occurs within the nucleus of the cell, without integration into the host DNA. Immediately after infection, the early viral genes (E1 to E4) are expressed, producing polypeptides that are important for regulation of gene expression, replication, and the inhibition of cellular apoptosis. Activation of the late genes results in expression of polypeptides required for encapsidation of the virus. At the end of the replication cycle, viruses are released by cell lysis (*see* ref. *73*).

Of many strains of adenoviruses, only strains 5 and 2 from the subgroup C *(74)* have been predominantly used to make vectors.

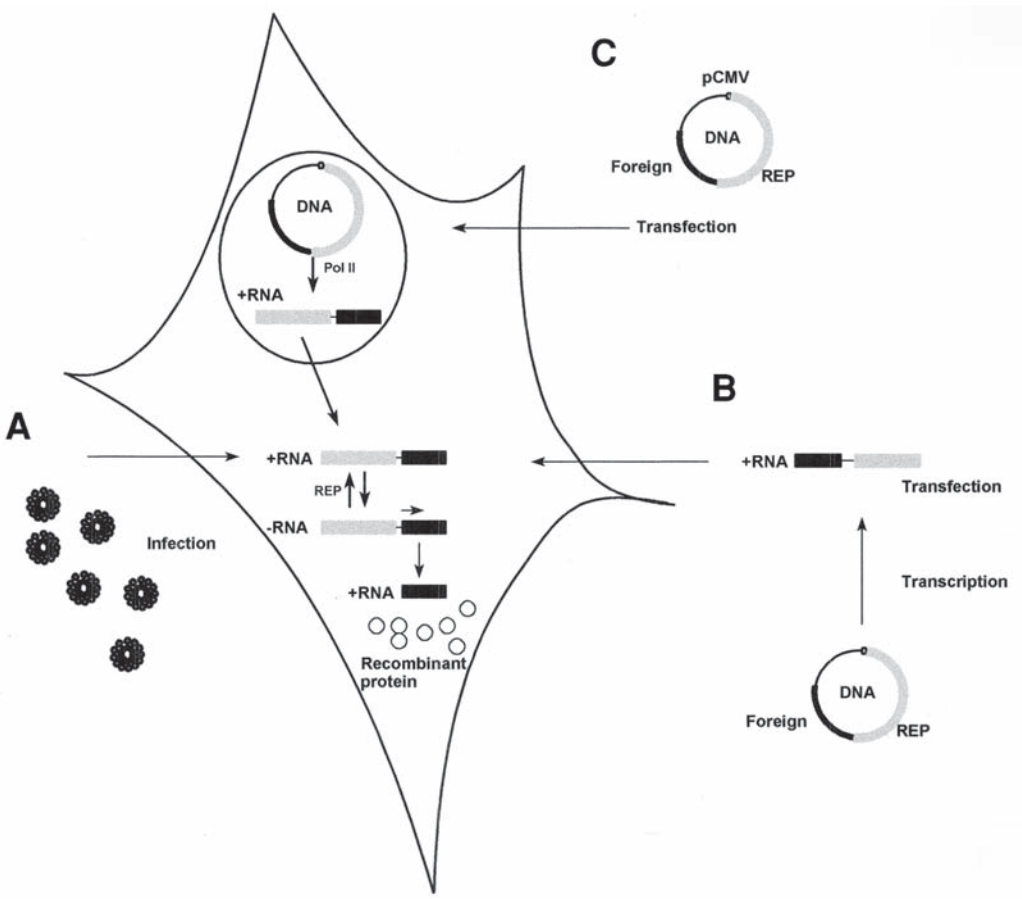

Fig. 4. Strategies of recombinant protein production using alphaviral vectors (adapted from ref. *72*). (**A**) Recombinant gene transferred by infection. The virus infects the cell by receptor-mediated endocytosis and the recombinant RNA that serves as mRNA is released and translated into the viral replicase. The replicase produces new full-length RNAs via a negative-strand template, which in turn serves as a template for the translation of the recombinant gene. (**B**) Plasmid DNA can be used for in vitro transcription of the viral mRNA, which is then transported directly to the cells. (**C**) An alternative is the transduction of a plasmid vector containing the viral replicase (REP) and the gene of interest. After transcription, recombinant viral RNA is transferred to the cytoplasm and amplified by the viral replicase and the gene product is translated.

Within the first-generation adenoviral vector, the E1 and E3 genes important for the activation of the viral early genes and for modulation of the immune response are replaced by the gene of interest. This leads to an insertion capacity of up to 8 kb. The E1/E3 replication-defective virus can be propagated in a cell line that complements E1, such as the human embryonic kidney-cell line 293 (Fig. 5). The transgene can be cloned into an adenoviral vector by recombination of a shuttle plasmid that contains the gene of interest and a second plasmid containing essentially the entire adenoviral genome in a circular form *(75)*, or by using a cosmid system.

More recently, defective adenovirus vectors were constructed in which all viral coding regions were removed, leaving only the inverted terminal repeats (ITR), the

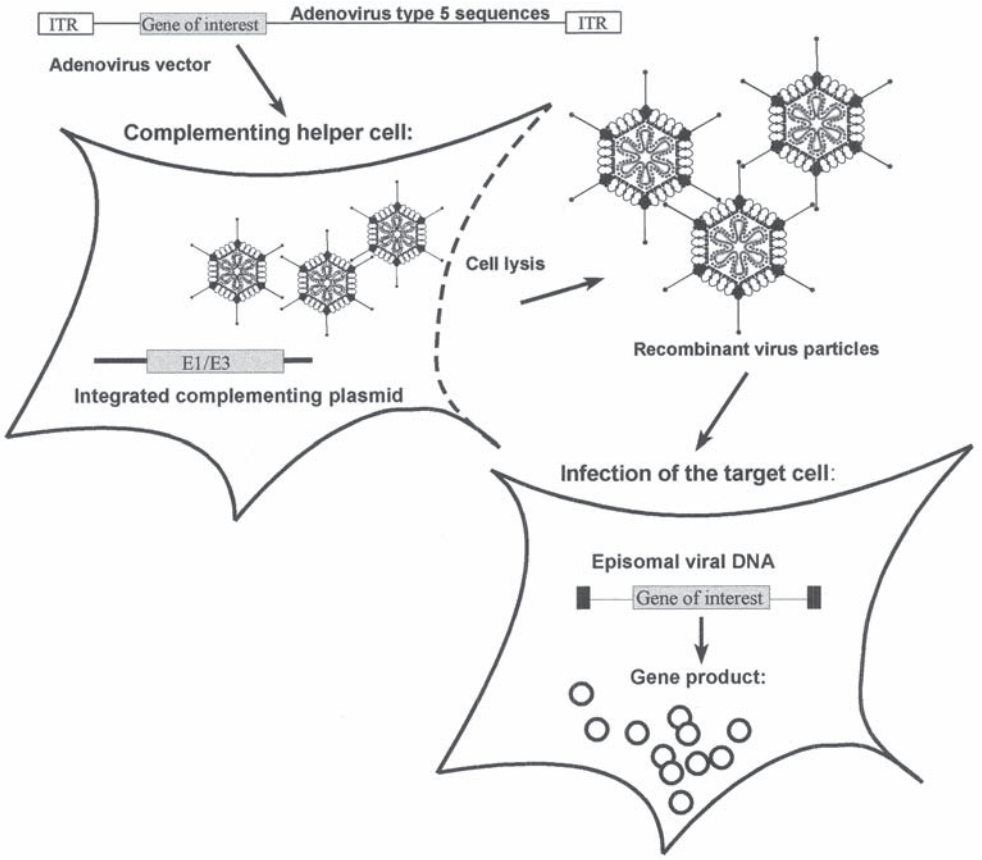

Fig. 5. Recombinant adenoviral vector production. The recombinant adenoviral vector is transferred to the complementing helper cell by conventional DNA transfection. In the course of viral amplification, the helper cells (often human embryonic kidney cell line 293) are lysed and the recombinant virus is harvested. After infection of a specific target cell, the linear DNA is transported to the nucleus and associated with the nuclear matrix. Because of the lack of chromosomal integration, expression of the transgene is only transient and decreases with the proliferation of the target cells.

transgene and the psi packaging sequences in these "gutless" vectors. Approximately 28 kb can be inserted. Such adenoviral vectors were successfully utilized for the expression of full-length genes such as the dystrophin gene and the cystic fibrosis transmembrane conductance regulator (CFTR) gene *(76,77)*. Since the production of gutless vectors depends on the co-expression of a helpervirus, strategies must be employed to separate both the helper and the recombinant virus carrying the transgene.

4.2. Viral Vectors for Stable Expression

A cell line that stably expresses a particular protein of interest is required in order to study the function of a given gene product or to continuously produce huge amounts of a biologically active protein. For this purpose, the retroviral transfer is a powerful tool.

4.2.1. Retroviruses

Retroviral vectors are effective transfer systems whenever stable introduction of foreign genes into target cells is needed. Recombinant retroviruses are efficiently used for the analysis of cDNA libraries *(78)*. Expression cloning is a powerful tool to isolate a cDNA of interest. Alternative techniques are based on transient expression of cDNA libraries in CHO cells which has obvious limitation in searches for proteins with various functions in specialized cell types, since the function of some proteins only become obvious during long-term expression. In contrast, retroviral gene transfer offers long term expression in a wide range of target cells, since the delivered transgene stably integrates into the host genome. This characteristic is also useful for fast and efficient establishment of target cells for high-throughput screening, and for validation of specific protein functions.

Retroviruses are RNA viruses that reverse-transcribe their diploid positive-stranded RNA genome into a double-stranded viral DNA, which is then stably inserted into the host DNA. Members of this class of RNA viruses are the murine leukemia viruses (MuLV) and the lentiviruses, which are extensively used for virus vector engineering. The advantage of the retroviral transfer system is its property of stable integration into the host genome, which leads to a stable expression of the gene of interest. Additionally, retroviruses possess the ability to infect a broad range of different cell types. Viral titers of up to 10^7 infectious particle per mL can be produced for efficient in vitro gene transfer. Moreover, single integration events can be easily established by using an appropriate ratio of virus particles and cells to be infected—multiplicity of infection (MOI). However, MLV-based vectors can only infect proliferating cells, because they need the breakdown of the nuclear membrane to deliver the preintegration complex into the cell nucleus.

The retroviral vectors currently mostly used for gene transfer are derived from the Moloney murine leukemia virus (MoMulV) (*see* ref. *79*). These viruses possess a relatively simple genome consisting of three structural genes—gag, pol, and env—responsible for replication, encapsidation, and infection. They are flanked by the viral long-terminal repeats (LTR), which are responsible for expression of the viral genome. By deleting the structural genes, but maintaining the *cis*-acting elements (LTR and packaging signal Psi) a space of 8 kb can be replaced by the gene of interest. For the production of virus, the recombinant vector is transduced into a packaging cell line that provides the viral proteins in trans. The recombinant viral RNA is packaged into viral particles and released from the helper cell. Packaging cell lines that yield titers of greater than 10^7 infectious viral particles per mL have been developed *(80)* (Fig. 6).

Because of the design of retroviral vectors and packaging cell lines, the formation of replication-competent retroviruses (RCRs) is avoided, thereby making this transfer applicable for various purposes including gene therapy *(81,82)*.

Typically, a selectable marker is co-inserted to isolate stably infected cells. A wide variety of vector constructions have been described that differ in size, orientation of the gene, and in the promoters used. In many retroviral vectors, the gene of interest is controlled by the viral LTR (LTR-based vectors). Additional internal promoters are used to express a second gene (internal promoter vectors). However, these vectors often suffer from reduced expression levels as a consequence of promoter interference *(83)*. An alternative to internal promoters offer the IRES leading to the co-expression of two

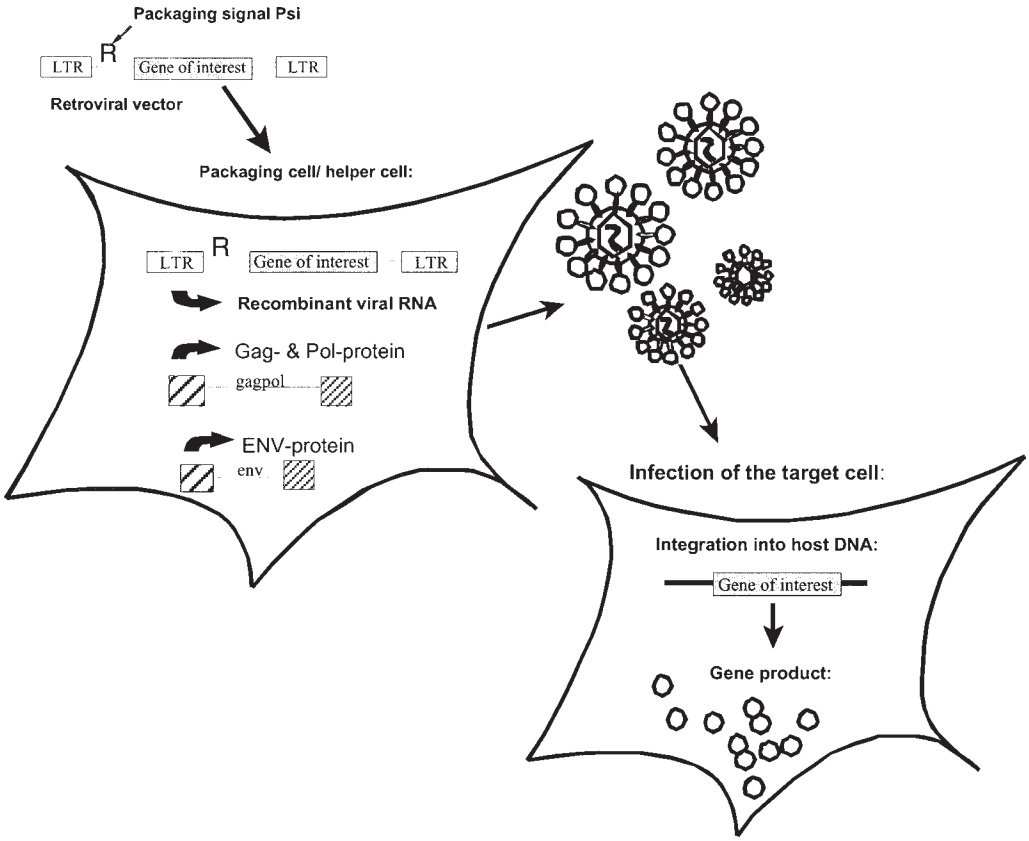

Fig. 6. Recombinant retroviral vector production. The recombinant retroviral DNA vector carrying the gene of interest is transferred into a helper cell that stably expresses the viral proteins. The transcribed genomic viral RNA is packaged into viral particles, which are released from the cell by a budding mechanism. After infection of the target cells the viral RNA genome is reverse-transcribed into DNA and integrated into the genome of the cell, leading to a stable expression of the protein.

genes from the LTR (*see* Chapter 3). A second possibility to circumvent promoter interferences is the use of retroviral self-inactivating vectors (SIN-vectors). In these vectors, the viral enhancer elements of the LTRs are deleted in infected cells *(84)*. SIN vectors are often used for transduction of regulated expression cassettes in order to reduce activating influences on the regulation properties of the transgene.

4.2.2. Stable Expression from Episomal Vectors

Some efforts have been made to construct vectors that replicate episomally in higher eukaryotic cells concomitant with the cell cycle. Although the expression of a stable integrated gene of interest is always influenced by the site of chromosomal integration, episomal expression offers a more predictable expression behavior *(85)*. A number of DNA viruses, such as SV40, BPV, or Epstein-Barr virus (EBV) replicate episomally in mammalian cells. This process depends on both viral transacting factors and accessory

activities recruited from the host-cell replication machinery. The viruses acquire centromer function by associating with the host chromosomes. Responsible for the episomal maintenance is a viral protein (EBNA-1 for EBV; large T-antigen for SV-40) that serves as replication and transcriptional enhancer.

EBV, as a herpesvirus with a double-stranded DNA genome (165 kb) is engineered to express large DNA fragments or entire authentic genes, including the native regulatory sequences of these genes for efficient gene expression in target cells *(86)*. The properties of EBNA-1 and oriP were successfully combined with the high and efficient adenoviral transfer system in order to extend the stability of expression of adenovirally transduced DNA. Therefore, the efficient adenoviral transfer system is used to deliver an EBV-based episome. After transduction, the episome is excised, circularized and thereby activated by the sequence-specific recombinase system Cre-loxP *(87)*. This technique extends expression time in proliferating cells; a loss of 2–5 % of vector copies occurs per cell division *(88,89)*

Another approach to achieve stable expression upon adenoviral gene transfer is the construction of chimeric adeno-retroviral vectors. The adenoviral part enables the efficient infection of proliferating and nonproliferating cells, whereas the retroviral part enhances the integration frequency of recombinant chimeric vectors into the genome of the target cells *(90,91)*. The adenoviral transfer of both the retroviral helper genes and a recombinant retroviral vector allows effective establishment of virus producers *in situ*. Recent developments could show that efficient integration does not require the retroviral helper genes *(91)*. Adeno-retroviral vectors free of any retroviral helper function are potent vectors for transduction and integration of regulatable expression cassettes (Unsinger, J., unpublished).

5. Metabolic Engineering of Mammalian Cells

The currently available cell lines for biotechnological use do not meet all criteria for optimal production of the protein of interest. The limitations may be essential for the quality of the protein or for the production process, yet the latter in turn often influences product quality. For this chapter, we have selected examples of metabolic engineering to demonstrate the problems and the principles of the current approaches.

5.1. Glycosylation Engineering

Post-translational processes were recognized as metabolic bottlenecks and potential targets to improve the quality of the products. This modification of a product protein includes various potentially rate-limiting interactions with proteins in the cytoplasm, the endoplasmatic reticulum (ER), and in the compartments of the Golgi apparatus. In many studies of mammalian cells the protein productivity and quality were improved by augmenting the post-translational capabilities. The major reason for using mammalian cell cultures for pharmaceutical protein production is the human-like post-translational modifications. Aside from protein processing, protein glycosylation is of central importance. Complex oligosaccharide side chains are covalently attached to newly synthesized peptide chains. The majority of secreted protein is glycosylated. Glycosylation can affect protein solubility, activity, and in vivo lifetime. The immune system recognizes nonhuman oligosaccharides as foreign, causing inflammatory and immune responses. For biopharmaceutical manufacture, the absence of sialic

(neuraminic) acid on the termini of complex carbohydrate structures—which results in more rapid clearance, as does the presence of high-mannose oligosaccharides—is of key importance.

N-linked oligosaccharides are transferred initially as a preformed core oligosaccharide from a dolichol lipid to the amide nitrogen of asparagine. In contrast, O-linked oligosaccharides are built starting with the attachment of N-acetylgalactosamine to the hydroxyl side chain of a serine or threonine residue. These initial steps are followed by a series of trimming or addition reactions in the ER and in the Golgi compartments. Glycoproteins are built up in a complex cascade of sequential, enzyme-catalyzed reactions and intracompartmental transport processes, often with multiple enzymes acting on common substrates to yield alternative oligosaccharide products *(92)*. The resulting glycosylation pattern of a protein is heterogeneous, and is highly dependent on multiple parameters such as primary amino acid sequence and protein conformation.

The glycosylation pattern can be partially altered by changing the culture medium composition, or by adding precursors, intermediates, or inhibitors of glycosylation *(93)*. Many of these reagents are toxic, and some alter glycosylation patterns inefficiently. For optimal glycosylation of therapeutic proteins, the choice of cell line plays a key role *(94)*. The human cell lines such as the 293 HEK line may be preferred, but may not be ideal. Producer-cell lines most widely used for pharmaceutical protein production, e.g., CHO and BHK cells, glycosylate recombinant proteins in a human-compatible way.

Recombinant-DNA technology for the metabolic engineering of glycosylation of mammalian cells aims to extend the host's oligosaccharide-biosynthesis capabilities by introducing genes that encode heterologous carbohydrate-synthesis enzymes. Glycosylation engineering strategies predominantly consider enzymes to be responsible for the terminal steps of complex oligosaccharide biosynthesis *(95–98)*. The following strategies are followed for this type of engineering: (i) glycosylation activities can be increased based either on gene activation or by expression of recombinant glycosylation genes; (ii) conversely, anti-sense RNA, ribozymes, or and other methods can be used to block undesirable glycosylation. Several glycosylation enzymes have been cloned and are available for metabolic engineering. Apart from analytical approaches, genetic engineering of cell lines aim to achieve more human-like glycosylation patterns and to improve the degree of terminal sialylation. Thus, CHO cells do not express α2,6-sialyltransferase (2,6 ST) *(99)*. Recombinant expression of a 2,6-ST gene results in a more human-like glycosylation profile, including both, α2,3- and α2,6-linked sialic acids *(96)*. Similarly, the glycosylation pattern in BHK cells has been successfully rendered more human-like by expression of α2,6-sialyltransferase and 1,3-fucosyltransferase-III genes.

Our understanding of the potential of glycosylation is incomplete. Although some general concepts have been clarified, important questions remain concerning activity and other relevant in vivo characteristics of individual glycoforms of a particular protein. Secreted protein products are a mixture of a number of different yet related isoforms. For clarification, these are purified and analyzed separately. These data serve as a basis for a rational engineering approach. Genes for enzymes involved in the important steps were identified, and are used for directed cell engineering. This process is intended to achieve pharmaceutical products with optimal in vivo characteris-

tics and to understand the involvement of glycoslyation in protein secretion and in the function of certain glycosylated forms of proteins.

5.2. Controlled Proliferation Technology

The control of cell growth and division is of fundamental importance for multicellular organisms. In the first developmental phase, cell proliferation is essential for growth of the organism. After terminal differentiation, the major growth phase is completed. Then, control of proliferation becomes the dominant aspect of the genetic stability of higher organisms. Nevertheless, the growth-arrested cells produce and secrete proteins continuously during their lifetime. This type of regulation is preferable in standard biotechnological production processes. However, for transformation of these processes, permanently proliferating animal cells are used. An ideal production process would include proliferation control to first allow cells to rapidly grow to high cell densities, followed by a proliferation-inhibited production phase in which the cells devote their metabolic capability to the formation of a product. Higher productivity of proliferation-inhibited cells was indeed observed with antibody-producing hybridoma cells *(100–102)*.

The first attempt to control cell proliferation by genetic engineering of the BHK cell line was based on an estrogen-regulated interferon-responsive factor 1 (IRF-1)-estrogen-receptor fusion *(103–107)*. IRF-1 is a DNA-binding transcription activator that accumulates in cells in response to interferons and has antiviral, antiproliferative, and anti-tumor activities *(108–112)*. Its function as a proliferation inhibitor relies on the induction of downstream genes *(104,109)*. Recombinant protein production can be strongly enhanced in IRF-1-arrested cells, by placing the transcription of the corresponding gene under the control of an IRF-1-responsive promoter *(113)*. In addition, expression of estrogen-responsive IRF-1 in a dicistronic configuration concomitant with the selection marker stabilizes the growth control in a way that allows several cycles of growth and growth-arrested states to be performed with the same culture *(107,114)*. Conditions to control cell growth and improve recombinant gene expression were extensively studied in BHK cells *(115,117)*. It was shown that the glycoslyation properties of the cells are not altered during the growth-inhibition phase *(118)*.

This genetic growth-control system can be used to facilitate the handling of helper cell lines in co-culture for cell and tissue engineering.To demonstrate this, the growth-control system was established in a human stroma feeder cell line. Transient estrogen exposure was sufficient to reduce cell growth for more than 1 wk. The proliferation-controlled cells could therefore be co-cultured with hematopoietic stem cells under conditions that are identical to those used with conventionally irradiation-arrested stroma cells. Analysis of the proliferation-controlled stroma cells revealed that the relevant characteristics of the parental L88/5 cells have been preserved in the regulated cell clone *(119)*.

The cyclin-dependent-kinase (cdk) inhibitors *p21* and *p27*, and the tumor-suppressor gene *p53* were used to reversibly arrest CHO cells at the G1-phase of the cell cycle *(120)*. Overexpression of these genes leads to a prevention of S-phase entry *(120)*. In the G1-phase of the cell cycle, cells check their physiological state and can arrest to repair genetic defects. In CHO cells *p21*, *p27*, and *p53* were expressed under tetracycline control. Transient expression of *p27* leads to growth arrest and gives rise to higher

specific protein productivity *(120)*. In contrast, continuous overexpression of *p53* leads to rapid cell death. Because the permanent expression of *p21* is not possible, tetracycline-regulated expression technology, in which the reporter SEAP and *p21* are co-expressed, was used. With this construct, growth arrest for several weeks and a significantly higher specific productivity was achieved when compared to control cells *(120)*. In other examples for controlled proliferation technology based on a *p27*-encoded expression unit, CHO cells were blocked in proliferation and reporter-gene expression was stimulated *(121)*.

5.3. Apoptosis Engineering

Apoptosis plays a fundamental role in multicellular life. In biotechnological production processes, apoptosis is an undesirable phenomenon, Processes are often limited by rapid cell death in the decline phase of a culture. Some commercially important production cell lines are sensitive to apoptosis, particularly hybridoma and myeloma cell lines *(122,123)*. In other cell lines, proliferation in response to nutrient limitations or genotoxic stress is blocked and no apoptosis is found. This allows the cells to replenish their metabolic precursors or repair DNA damage *(124)*.

Apart from the elimination of nutrient deprivation by feeding strategies, chemical-medium additives are used to block apoptosis pathways. Suppression of apoptosis in cell-culture processes is applied to engineer the cells using antiapoptotic genes. Mammalian cells were shown to be successfully protected against stress-induced apoptosis by overexpression survival genes from the bcl-2 *(125)*.

The level of protection varies between different cell types and cell lines and, in most cases, Bcl-2 overexpression cannot prevent cell death, but it can extend cellular lifetime and lead to increased production *(102,125–127)*. Attempts to complement the action of Bcl-2 using concomitant overexpression of the anti-apoptotic genes bag-1, bcl-x_L or the adenoviral E1B-19K gene were encouraging and showed that the protective effects of individual genes were equal or even additive when, for example, Bag-1 and Bcl-2 were co-expressed *(128–131)*. More recent strategies to prevent apoptosis concern the inhibition of caspases. These are downstream actors of the programmed cell death cascade. Using peptide inhibitors and the expression of genes encoding dominant negative caspases, effective inhibition of apoptosis could be observed *(132)*.

References

1. Hauser, H. and Wagner, R., eds. (1997) *Mammalian Cell Biotechnology in Protein Production*, W. DeGruyter, New York, pp. 1–190.
2. Kozak M. (1999) Initiation of translation in prokaryotes and eukaryotes. *Gene* **234**, 187–208.
3. Gossen, M. and Bujard, H. (1992) Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc. Natl. Acad. Sci. USA* **89**, 5547–5551.
4. Gossen, M., Freundlieb, S., Bender, G., Müller, G., Hillen, W., and Bujard, H. (1995) Transcriptional activation by tetracyclines in mammalian cells. *Science* **268**, 1766–1769.
5. Shockett, P. E., Difilippantonio, M., Hellman, N., and Schatz, D. G. (1995) A modified tetracycline-regulated system provides autoregulatory, inducible gene expression in cultured cells and transgenic mice. *Proc. Natl. Acad. Sci. USA* **92**, 6522–6526.
6. A-Mohammadi, S. and Hawkins, R. E. (1998) Efficient transgene regulation from a single tetracycline-controlled positive feedback regulatory system. *Gene Ther.* **5**, 76–84.

7. Strathdee, C. A., McLeod, M. R., and Hall, J. R. (1999) Efficient control of tetracycline-responsive gene expression from an autoregulated bi-directional expression vector. *Gene* **229**, 21–29.
8. Unsinger, J., Kröger, A., Hauser, H., and Wirth, D. (2001) Retroviral vectors for transduction of an autoregulated bidirectional expression cassette. *Mol. Ther.* **4**, 484–489.
9. Fussenegger, M., Morris, R. P., Fux, C., Rimann, M., von Stockar, B., Thompson, C. J., et al. (2000) Streptogramin-based gene regulation systems for mammalian cells. *Nat. Biotechnol.* **18**, 1203–1208.
10. Eilers, M., Picard, D., Yamamoto, K. R., and Bishop, J. (1989) Chimaeras of myc oncoprotein and steroid receptors cause hormone-dependent transformation of cells. *Nature* **340**, 66–68.
11. Braselmann, S., Graninger, P., and Busslinger, M. (1993) A selective transcriptional induction system for mammalian cells based on Gal4-estrogen receptor fusion proteins. *Proc. Natl. Acad. Sci. USA* **90**, 1657–1661.
12. Wang, Y., O'Malley, Jr., B. W., Tsai, S. Y., and O'Malley, B. W. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 8180–1884.
13. Burcin, M. M., O'Malley, B. W., and Tsai, S. Y. (1998) A regulatory system for target gene expression. *Front. Biosci.* **3**, 1–7.
14. No, D. Yao, T. P., and Evans, R. M. (1996) Ecdysone-inducible gene expression in mammalian cells and transgenic mice. *Proc. Natl. Acad. Sci. USA* **93**, 3346–3351.
15. Kaufman, R. (1990) Vectors used for expression in mammalian cells. *Methods Enzymol.* **185**, 487–512.
16. Boshart, M., Weber, F., Jahn, G., Dorsch-Häsler, K., Fleckenstein, B., and Schaffner, W. (1985) A very strong enhancer is located upstream of an immediate early gene of human cytomegalovirus. *Cell* **41**, 521–530.
17. Bell, A. C. and Felsenfeld, G. (2001) Gene regulation: insulators and boundaries: versatile regulatory elements in the eukaryotic genome. *Science* **291**, 447–450.
18. Bode, J., Benham, C., Knopp, A., and Mielke, C. (2000) Transcriptional augmentation: modulation of gene expression by Scaffold/matrix attached regions (S/MAR elements). *Crit. Rev. Eukaryot. Gene Expr.* **10**, 73–90.
19. Fiering, S., Whitelaw, E., and Martin, D. I. K. (2000) To be or not to be active: the stochastic nature of enhancer action. *Bioessays* **22**, 381–387.
20. Ogbourne, S. and Antalis, T. M. (1998) Transcriptional control and the role of silencers in transcriptional regulation in eukaryotes. *Biochem. J.* **331**, 1–14.
21. Bode, J., Schlake, M., Ríos-Ramírez, M., Mielke, C., Stengert, M., Kay, V., et al. (1995) Scaffold/matrix-attached regions: structural properties creating transcriptioally active loci, in *Structural and Functional Organization of the Nuclear Matrix (International Review of Cytology)*. (Berezney, R. and Jeon, K. W., eds.), Academic Press, San Diego, CA, pp. 389–453.
22. Bell, A. C. and Felsenfeld, G. (1999) Stopped at the border: boundaries and insulators. *Curr. Opin. Genet. Dev.* **9**, 191–198.
23. Li, Q. L., Harju, S., and Peterson, K. R. (1999) Locus control regions – coming of age at a decade plus. *Trends Genet.* **15**, 403–408.
24. Dorner, A. J., Wasley, L. C., and Kaufman, R. J. (1989) Increased synthesis of secreted proteins induces expression of glucose- regulated proteins in butyrate-treated Chinese hamster ovary cells. *J. Biol. Chem.* **264**, 20,602–20,607.
25. Kaufman, R. J., Wasley, L. C., Spiliotes, A. J., Gossels, S. D., Latt, S. A., Larsen, G. R., et al. (1985) Coamplification and coexpression of human tissue-type plasminogen activator and murine dihydrofolate reductase sequences in Chinese hamster ovary cells. *Mol. Cell. Biol.* **5**, 1750–1759.
26. Looney, J. E. and Hamlin, J. L. (1987) Isolation of the amplified dihydrofolate reductase domain from methotrexate-resistant Chinese hamster ovary cells. *Mol. Cell. Biol.* **7**, 569–577.

27. Brown, M, E., Renner, G., Field R, P., and Hassell, T. (1992) Process development for the production of recombinant antibodies using the glutamine-synthetase (GS) system. *Cytotechnology* **9**, 231–236.
28. Cappechi, M. R. (1990) Gene targeting: how efficient can you get? *Nature* **348**, 109.
29. Kilby, N. J., Snaith, M. R., and Murray, J. A. H. (1993) Site-specific recombinases: tools for genome engineering. *Trends Genet.* **9**, 413–421.
30. Baer, A. and Bode, J. (2001) Coping with kinetic and thermodynamic barriers: RMCE, an efficient strategy for the targeted integration of transgenes. *Curr. Opin. Biotechnol.* **12**, 473–480.
31. Klehr-Wirth, D., Kuhnert, F., and Hauser, H. (1997) Generation of mammalian cells with conditional expression of cre recombinase. *Technical Tips online*, T40067., URL: http://tto.trends.com.
32. Kühn, R., Rajewski, K., and Müller, W. (1995) Inducible gene targeting in mice. *Science* **269**, 1427.
33. Metzger, D., Clifford, J., Chiba, H., and Chambon, P. (1995) Conditonal site-specific recombination in mamalian cells using a ligand-dependent chimeric Cre recombinase. *Proc. Natl. Acad. Sci. USA* **92**, 6991–6995.
34. Kellendonk, D., Tronche, F., Monaghan, A. P., Angrand, P. O., Stewart, F., and Schütz, G. (1996) Regulation of cre recombinase activity by the synthetic steroid RU486. *Nucleic Acids Res.* **24**, 1404–1411.
35. Schlake, T. and Bode, J. (1994) Use of mutated Flp recognition target (FRT) sites for the exchange of expression cassettes at defined chromosomal loci. *Biochemistry* **33**, 12,746–12,751.
36. Lee, G., Kim, S., Lee, G. K. M., and Park, J. (2000) An engineered lox sequence containing part of a long terminal repeat of HIV-1 permits cre recombinase-mediated excision. *Biochem. Cell Biol.* **78**, 653–658.
37. Seibler, J. and Bode, J. (1997) Double-reciprocal crossover mediated by Flp recombinase. A concept and an assay. *Biochemistry* **36**, 1740–1747.
38. Seibler, J., Schübeler, D., Fiering, S., Groudine, M., and Bode, J. (1998) DNA cassette exchange in ES cells mediated by Flp recombinase: an efficient strategy for repeated modification of tagged loci by marker-free constructs. *Biochemistry* **37**, 6229–6234.
39. Verhoeyen, E., Hauser, H., and Wirth, D. (1998) Efficient targeting of retrovirally FRT-tagged chromosomal loci. Techn. Tips online T01515 URL: http://tto.trends.com.
40. Schübeler, D., Maass, K., and Bode, J. (1998) Retargeting of retroviral integration sites for the pedictable expression of transgenes and the analysis of cis-acting sequences. *Biochemistry* **37**, 11,907–11,914.
41. Verhoeyen, E., Hauser, H., and Wirth, D. (2001) Evaluation of retroviral vector design in defined chromosomal loci by Flp-mediated cassette replacement. *Hum. Gene Ther.* **12**, 933–944.
42. Müller, P., Oumard, A., Wirth, D., Kröger, A., and Hauser, H. (2001) Polyvalent vectors for coexpression of multiple genes, in *Plasmids for Therapy and Vaccination* (Schleef, M., ed.), Wiley-VCH, Weinheim, pp. 119–136.
43. Martinez-Salas, E. (1999) Internal ribosome entry site biology and its use in expression vectors. *Curr. Opin. Biotechnol.* **10**, 458–464.
44. Jackson, R. J. and Kaminski, A. (1995) Internal initiation of translation in eukaryotes: the picornavirus paradigm and beyond. *RNA* **1**, 985–1000.
45. Sachs, A. B., Sarnow, P., and Hentze, M. W. (1997) Starting at the beginning, middle, and end: translation initiation in eukaryotes. *Cell* **89**, 831–838.
46. Pelletier, J. and Sonenberg, N. (1988) Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA. *Nature* **334**, 320–325.
47. Pestova, T. V., Hellen, C. U. T., and Shatsky, I. N. (1996) Canonical eukaryotic initiation factors determine initiation of translation by internal ribosome entry. *Mol. Cell. Biol.* **16**, 6859–6869.

48. Jang, S. K., Krausslich, H. G., Nicklin, M. J., Duke, G. M., Palmenberg, A. C., and Wimmer, E. (1988) A segment of the 5' nontranslated region of encephalomyocarditis virus RNA directs internal entry of ribosomes during in vitro translation. *J. Virol.* **62**, 2636–2643.
49. Oumard, A., Hennecke, M., Hauser, H., and Nourbakhsh, M. (2000) Translation of NRF mRNA is mediated by highly efficient internal ribosome entry. *Mol. Cell. Biol.* **20**, 2755–2759.
50. Chappell, S. A., Edelman, G. M., and Mauro, V. P. (2000) A 9-nt segment of a cellular mRNA can function as an internal ribosome entry site (IRES) and when present in linked multiple copies greatly enhances IRES activity. *Proc. Natl. Acad. Sci. USA* **97**, 1536–1541.
51. Dirks, W., Schaper, F., Kirchhoff, S., Morelle, C., and Hauser, H. (1994) A multifunctional vector family for gene expression in mammalian cells. *Gene* **149**, 387–388.
52. Dirks, W., Wirth, M., and Hauser, H. (1993) Dicistronic units for gene expression in mammalian cells. *Gene* **128**, 247–249.
53. Zitvogel, L., Tahara, H., Cai, Q., Storkus, W. J., Muller, G., Wolf, S. F., et al. (1994) Construction and Characterization of retroviral vectors expressing biologically active human interleukin-12. *Human Gene Ther.* **5**, 1493–1506.
54. Fussenegger, M., Mazur, X., and Bailey J. E. (1998) pTRIDENT, a novel vector family for tricistronic gene expression in mammalian cells. *Biotechnol. Bioeng.* **51**, 1–10.
55. Schirmbeck, R., von Kampen, J., Metzger, K., Wild, J., Grüner, B., Schleef, M., et al. (1999) DNA-based vaccination with polycistronic expression plasmids. *Methods in Molecular Medicine* **29**, 313–322.
56. Kwissa, M., Unsinger, J., Schirmbeck, R., Hauser, H., and Reimann, J. (2000) Polyvalent DNA vaccines with bidirectional promoters. *J. Mol. Med.* **78**, 495–506.
57. Mielke, C., Tümmler, M, Schübeler, D., von Hoegen, I., and Hauser, H, (2000) Stabilized, long-term expression of heteromeric proteins from tricistronic mRNA. *Gene* **254**, 1–8.
58. Attal, J., Theron, M. C., and Houdebine, L. M. (1999) The optimal use of IRES (internal ribosome entry site) in expression vectors, *Genet. Anal.* **15**, 161–165.
59. Hennecke, M., van Kampen, J., Metzger, K., Schirmbeck, R., Reimann, J., and Hauser, H. (2001) The strength of IRES-driven translation of bicistronic expression vectors depends on the composition of the mRNA. *Nucleic Acids Res.* **29**, 3327–3334.
60. Baron, U., Freundlieb, S., Gossen, M., and Bujard, H. (1995) Co-regulation of two gene activities by tetracycline via a bidirectional promoter. *Nucleic Acids Res.* **23**, 3605–3606.
61. Liljestrom, P. (1994) Alphavirus expression systems. *Curr. Opin. Biotechnol.* **5**, 495–500.
62. Johnston, R. E. and Peters, C. J. (1996) Alphaviruses, in *Fields Virology* (Fields B. N., Knipe D. M., Howley P. M., eds.), Lippincott-Raven: Philadelphia, PA, pp. 842–898.
63. Strauss, J. H. and Strauss, E. G. (1994) The alphavirus: gene expression, replication and evolution. *Microbiol. Rev.* **58**, 491–562.
64. Pushko, P., Parker, M., Ludwig, G. V., Davis, N. L., Johnston, R. E., and Smith, J. L. (1997) Replicon helper systems from attenuated Venezuelan equine encephalitis virus: expression of heterologous genes in vitro and immunization against heterologous heterologous genes in vitro and immunization against heterologous pathogens in vivo. *Virology* **39**, 389–401.
65. Berglund, P. M., Sjoberg, M., Garoff, H., Atkins, G. J., Sheahan, B. J., and Liljestrom, P. (1993) Semiliki Forest virus expression systems: production of conditionally infectious recombinant particles. *Bio-technology* **11**, 916–920.
66. Schlesinger, S. (1993) Alphaviruses-vectors for the expression of heterologous genes. *Trends Biotechnol.* **11**, 18–22.
67. Schlesinger, S. (1995) RNA viruses as vectors for the expression of heterologous proteins. *Mol. Biotechnol.* **3**, 155–165.
68. Wahlfors, J. J., Zullo, S. A., Nelson, D. M., and Morgan, R. A. (2000) Evaluation of recombinant alphaviruses as vectors in gene therapy. *Gene Ther.* **7**, 472–480.

69. Herweijer, H., Latendresse, J. S., Williams, P., Zhang, G., Danko, J., Schlesinger, S., et al. (1995) A plasmid-based self amplifying [Sindbis] virus vector. *Hum. Gene Ther.* **6**, 1161–1167.
70. Dubensky, T.W., Driver, D. A., Polo, J. M., Belli, B. A., Latham, E. M., Ibnaez, C. E., et al. (1996) Sindbis virus DNA-based expression vectors: utility for in vitro and in vivo gene transfer. *J. Virol.* **70**, 508–519.
71. Perri, S., Driver, D. A., Gardner, J. P., Sherrill, S., Belli, B. A., Dubensky, T. W., et al. (2000) Replicon vectors derived from Sindbis Virus and Semliki forest virus that establish persistent replication in host cells. *J. Virol.* **74**, 9802–9807.
72. Tubulekas, I., Berglund, P., Fleeton, M., and Liljeström, P. (1997) Alphavirus expression vectors and their use as recombinant vaccines: a minireview. *Gene* **190**, 191–195.
73. Horwitz, M. S. (1996) Adenoviruses, in *Fields Virology* (Fields, B. N., Knipe, D. M., and Howley, P. M., eds.), Lippincott-Raven: Philadelphia, PA, pp. 2149–2171
74. Romano, G., Micheli, P., Pacilio, C., and Giordano, A. (2000) Latest developments in gene transfer technology: achievments, perspectives, and controversies over therapeutic applications. *Stem Cells* **18**, 19–39.
75. Hitt, M., Bett, A. J., Prevec, L., and Graham, F. L. (1994) Construction and propagation of human adenovirus vectors, in *Cell Biology: A Laboratory Handbook*. Academic Press, San Diego, CA, pp. 479–490.
76. Kochanek, S., Clemens, P. R., Mitani, K., Chen, H. H., Chan, S., and Caskey, C. T. (1996) A new adenoviral vector: replacement of all viral coding sequences with 28 kb of DNA independently expression both full length dystrophin and ß-galactosidase. *Proc. Natl. Acad. Sci. USA* **93**, 5731–5736.
77. Fisher, K. J., Choi, H., Burda, J., Chen, S. J., and Wilson, J. M. (1996) Recombinant adenovirus deleted of all viral genes for gene therapy of cystic fibrosis. *J. Virol.* **217**, 11–22.
78. Kitamura, T., Onishi, M., Kinoshita, S., Shibuya, A., Miyajima, A., and Nolan, G. P. (1995) Efficient screening of retroviral cDNA expression libraries. *Proc. Natl. Acad. Sci. USA* **92**, 9146–9150.
79. Walther, W. and Stein, U. (2000) Viral vectors for gene transfer: a review of their use in the treatment of human diseases. *Drugs* **60**, 249–271.
80. Cosset, F. L., Takeuchi, Y., Battini, J. L., Weiss, R. A., and Collins, M. K. (1995) High titer packaging cells producing recombinant retroviruses resistant to human serum. *J. Virol.* **69**, 7430–7436.
81. Chong, H. and Vile, R. C. (1996) Replication-competent retrovirus produced by a "split-function" third generation amphotropic packaging cell line. *Gene Ther.* **3**, 624–629.
82. Palù, G., Parolin, C., Takeuchi, Y., and Pizzato, M. (2000) Progress with retroviral gene vectors. *Rev. Med. Virol.* **10**, 185–202.
83. Emerman, M., and Temin, H. M. (1986) Comparison of a promoter suppression in avian and murine retrovirus vectors. *Nucleic Acids Res.* **14**, 9381–9396.
84. Yu, S. F., von Ruden, T., Kantoff, P. W., Garber, C., Seiberg, M., Ruther, U., et al. (1995) Self-inactivating retroviral vectors designed for transfer of whole genes into mammalian cells. *Bio-technology* **13**, 389–392.
85. Bode, J., Fetzer, C. P., Nehlsen, K., Scinteie, M., Hinrichsen, B., Baiker, A., et al. (2001) The hitchhiking principle: Optimization episomal vectors for the use in gene therapy and biotechnology. *Gene Ther. Mol. Biol.* **6**, 33–46.
86. Van Craenenbroeck, K., Vanhoenacker, P., Duchau, H., and Haegeman, G. (2000) Molecular integrity and usefulness of episomal expression vectors derived form BK and Epstein-Barr virus. *Gene* **253**, 293–301.
87. Tan, B. T., Wu, L., and Berk, A. (1999) An Adenovirus–Epstein–Barr virus hybrid vector that stably transforms cultured cells with high efficiency. *J. Virol.* **73**, 7582–7589.

88. Leblois, H., Roche, C., Falco, D., Orsini, P., Yeh, P., and Perricaudet, M. (2000) Stable transduction of actively dividing cells via a novel adenoviral/episomal vector. *Molecular Therapy* **1**, 314–322.
89. Krougliak, V. A., Krougliak, N., and Eisensmith, R. C. (2000) Stabilization of transgenes delivered by recombinant adenovirus vectors through extrachromosomal replication. *J. Gene. Med.* **3**, 51–58.
90. Feng, M., Jackson, W. H., Goldman, C. K., Rancourt, C., Wang, M., Dusing, S. K., et al. (1997) Stable in vivo gene transduction via a novel adenoviral/retroviral chimeric vector. *Nat. Biotechnol.* **15**, 866–870.
91. Zheng, C., Baum, B. J., Iadarola, M. J., and O'Connell, B. C. (2000) Genomic integration and gene expression by a modified adenoviral vector. *Nat. Biotechnol.* **18**, 176–180.
92. Umaña, P. and Bailey, J. E. (1997) A mathematical model of N–linked glycoform biosynthesis. *Biotechnol. Bioeng.* **55**, 890–908.
93. Minch, S. L., Kallio, P. T., and Bailey, J. E. (1995) Tissue plasminogen activator coexpressed in Chinese hamster ovary cells with alpha(2,6)–sialyltransferase contains NeuAc alpha(2,6) Gal beta(1,4)Glc–N–AcR linkages. *Biotechnol. Prog.* **11**, 348–351.
94. Elbein, A. (1991) Glycosidase inhibitors: inhibitors of N–linked oligosaccharide processing. *FASEB J.* **5**, 3055–3063.
95. Grabenhorst, E., Costa, J., and Conradt, H. S. (1997) in *Animal Cell Technology* (Carrondo, M. J. T., Griffiths, B., and Moreira, J. L. P., eds.), Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 481–487.
96. Grabenhorst, E., Hoffmann, A., Nimtz, M., Zettlmeissl, G., and Conradt, H. S. (1994) Construction of stable BHK-21 cells coexpressing human secretory glycoproteins and human Gal(beta 1-4)GlcNAc–R alpha 2,6-sialyltransferase alpha 2,6-linked NeuAc is preferentially attached to the Gal(beta 1-4)GlcNAc(beta 1-2)Man(alpha 1-3)–branch of diantennary oligosaccharides from secreted recombinant beta–trace protein. *Eur. J. Biochem.* **232**, 718–725.
97. Sburlati, A., Umaña, P., Prati, E. G. P., and Bailey, J. E. (1998) Synthesis of bisected glycoforms of recombinant IFN–beta by overexpression of beta-1,4-N-acetylglucosaminyltransferase III in Chinese hamster ovary cells. *Biotechnol. Prog.* **14**, 189–192.
98. Li, E., Gibson, R., and Kornfeld, S. (1980) Structure of an unusual complex-type oligosaccharide isolated from Chinese hamster ovary cells. *Arch. Biochem. Biophys.* **199**, 393–399.
99. Costa, J., Grabenhorst, E., Nimtz, M., and Conradt, H. S. (1996) Stable expression of the Golgi form and secretory variants of human fucosyltransferase III from BHK-21 cells. Purification and characterization of an engineered truncated form from the culture medium. *J. Biol. Chem.* **272,** 11,613–11,621.
100. Suzuki, E. and Ollis, D. F. (1990) Enhanced antibody production at slowed growth rates: experimental demonstration and a simple structured model. *Biotechnol. Prog.* **6**, 231–236.
101. Franek, F. and Dolnikova, J. (1991) Hybridoma growth and monoclonal antibody production in iron-rich protein-free medium: effect of nutrient concentration. *Cytotechnology* **7**, 33–38.
102. Al-Rubeai, M., Emery, A. N., Chalder, S., and Jan, D. C. (1992) Specific monoclonal antibody productivity and the cell cycle–comparisons of batch, continuous and perfusion cultures. *Cytotechnology* **9**, 85–97.
103. Kirchhoff, S., Schaper, F., and Hauser, H. (1993) Interferon regulatory factor 1 (IRF-1) mediates cell growth inhibition by transactivation of downstream target genes. *Nucl. Acids Res.* **21**, 2881–2889.
104. Kirchhoff, S., Koromilas, A., Schaper, F., Grashoff, M., Sonenberg, N., and Hauser, H. (1995) IRF-1 induced cell growth inhibition and interferon induction requires the activity of the protein kinase PKR. *Oncogene* **11**, 439–445.
105. Köster, M., Kirchhoff, S., Schaper, F., and Hauser, H. (1995) Proliferation control of mammalian cells by the tumor suppressor IRF–1. *Cytotechnology* **18**, 67–75.

106. Köster, M., Kirchhoff, S., Schaper, F., and Hauser, H. (1995) Proliferation control of mammalian cells by the tumor suppressor IRF-1, in *Animal Cell Technology: Developments Towards the 21st Century* (Beuvery, C., Griffiths, B., and Zeijlemaker, W. P., eds.) Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 33–44
107. Carvalhal, A. V., Moreira, J. L., Müller, P. P., Hauser, H., and Carrondo, M. J. T. (1998) Cell growth inhibition by the IRF-1 system, in *New Developments and New Applications in Animal Cell Technology* (Merten, O.-W., Perrin, P, and Griffiths, B., eds.), Kluwer Academic Publishers, pp. 215–217.
108. Schaper, F., Kirchhoff, S., Posern, G., Köster, M. Oumard, A., Sharf, R., et al. (1998) Functional domains of Interferon Regulatory Factor 1 (IRF-1). *Biochemical J.* **335**, 147–157.
109. Kirchhoff, S., Wilhelm, D., Angel, P., and Hauser, H. (1999) NFκB activation is required for IRF-1 mediated IFN-β. *Eur. J. Biochem.* **261**, 546–554.
110. Kirchhoff, S., Oumard, A., Nourbakhsh, M., Levi, B.-Z., and Hauser, H. (2000) Interplay between repressing and activating domains defines the transcriptional activity of IRF–1. *Eur. J. Biochem.* **267**, 6753–6761.
111. Müller, P. P., Carvalhal, A. V., Moreira, J. L., Geserick, C., Schroeder, K., Carrondo, M. J. T., et al. (1999) Development of an IRF-1-based proliferation control system, in *Cell Engineering 1* (Al-Rubeai, M., Betenbaugh, M., Hauser, H., Jenkins, N., MacDonald, C., Merten, A.-W., eds.), Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 220–238.
112. Kröger, A., Köster, M., Schroeder, K., Hauser, H., and Mueller, P. P. (2002) Activities of IRF-1. *J. Interferon and Cytokine Res.* **22,** 5–14.
113. Geserick, C., Bonarius, H. P. J., Kongerslev, L., Hauser, H., and Müller, P. P. (2000) Enhanced productivity during controlled proliferation of BHK cells in continuously perfused bioreactors. *Biotech. Bioeng.* **69**, 266–274.
114. Müller, P. P., Kirchhoff, S., and Hauser, H. (1998) Sustained expression in proliferation controlled BHK–21 cells, in New developments and new applications in animal cell technology (Merten, O.-W., Perrin, P, and Griffiths, B., eds.), Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 209–214.
115. Kirchhoff, S., Kröger, A., Cruz, H., Tümmler, M., Schaper, F., Köster, M., et al. (1996) Regulation of cell growth by IRF-1 in BHK-21 cells. *Cytotechnology* **22**, 147–156.
116. Geserick, C., Schroeder, K., Bonarius, H., Kongerslev, L., Schlenke, P., Hauser, H., et al. (1999) Recombinant pharmaceutical protein overexpression in an IRF-1 proliferation controlled production system, in *Animal Cell Technology: Products from Cells, Cells as Products* (Bernard, A., Griffiths, B., Noé, W., and Wurm, F. eds.), Kluwer Academic Publishers, The Netherlands, pp. 3–10.
117. Carvalhal, A. V., Moreira, J. L., Cruz, H., Mueller, P. P., Hauser, H., and Carrondo, M. J. T. (2000) Manipulation of culture conditions for BHK cell growth inhibition by IRF-1 activation. *Cytotechnology* **32**, 135–145.
118. Müller, P. P., Grabenhorst, E., Conradt, H. S., and Hauser, H. (1999) Recombinant glycoprotein product quality in proliferation controlled BHK-21 cells. *Biotechnol. Bioeng.* **65**, 529–536.
119. Schroeder, K., Koschmieder, S., Ottmann, O. G., Hoelzer, D., Hauser, H., and Mueller, P. P. (2002) Genetic proliferation control facilitates the handling of a human stromal feeder cell line during coccultivation with hematopoietic progenitor cells. *Biotechnol. Bioeng.* **78,** 346–352.
120. Fussenegger, M., Schlatter, S., Dätwyler, D., Mazur, X., and Bailey, J. E. (1998) Controlled proliferation by multigene metabolic engineering enhances the productivity of Chinese hamster ovary cells. *Nat. Biotechnol.* **16**, 468–472.
121. Fussenegger, M. and Bailey, J. E. (1998) Molecular regulation of cell-cycle progression and apoptosis in mammalian cells: implication for biotechnology. *Biotechnol. Prog.* **14**, 807–833.

122. Mercille, S. and Massie, B. (1999) Apoptosis-resistant E1B-19K-expressing NS/0 myeloma cells exhibit increased viability and chimeric antibody productivity under perfusion culture conditions. *Biotechnol. Bioeng.* **63**, 529–543.
123. Cotter, T. G. and Al-Rubeai, M (1995) Cell death (apoptosis) in cell culture systems. *Trends Biotechnol.* **13**, 150–155.
124. Perreault, J. and Lemieux, R. (1993) Essential role of optimal protein synthesis in preventing the apoptotic death of cultured B cell hybridomas. *Cytotechnology* **13**, 99–105.
125. Singh, R. P., Emery, A. N., and Al-Rubeai, M. (1996) Enhancement of survivability of mammalian cells by overexpression of the apoptosis-suppressor gene bcl-2. *Biotechnol. Bioeng.* **52**, 166–175.
126. Chung, J. D., Sinskey, A. J., and Stephanopoulos, G. (1998) Growth factor and bcl-2 mediated survival during abortive proliferation of hybridoma cell line. *Biotechnol. Bioeng.* **57**, 164–171.
127. Simpson, N. H., Milner, A. E., and Al-Rubeai, M. (1997) Prevention of hybridoma cell death by bcl-2 during suboptimal culture conditions. *Biotechnol. Bioeng.* **54**, 1–16.
128. Huang, D. C. S., Cory, S., and Strasser, A. (1997) Bcl-2, Bcl-XL and adenovirus protein E1B19kD are functionally equivalent in their ability to inhibit cell death. *Oncogene* **14**, 405–414.
129. Terada, S., et al. (1997) Anti-apoptotic genes, bag-1 and bcl-2, enabled hybridoma cells to survive under treatment for arresting cell cycle. *Cytotechnology* **25**, 17–23.
130. Zanghi, J. A., Fussenegger, M., and Bailey, J. E. (1999) Serum protects protein–free competent Chinese hanster ovary cells against apoptosis induced by nutrient deprivation in batch culture. *Biotechnol. Bioeng.* **5**, 573–582.
131. Mastrangelo, A. J., Hardwick, M. J. Zou, S., and Betenbaugh, M. J. (2000) Part 2. Overexpression of bcl-2 family members enhances survival of mammalian cells in response to various culture insults. *Biotechnol. Bioeng.* **67**, 555–564.
132. Vives, J., Juanola, S., Gabernet, C., Prats, E., Cairó, J. J., Cornudella, L., et al. (2001) Genetic strategies for apoptosis protection and hybridoma cells based on overexpression of cellular and viral proteins, in *Animal Cell Technology: From Target to Market* (Lindner-Olsson, E., et al., eds.), Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 230–233.