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Development of a Protein-Free Protocol for Cell Line Generation and Investigation of Genetic Parameters: Early Clone Screening favours the Selection of Stable Production Clones

Dissertation

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Eingereicht von Dipl.-Ing. Christine Lattenmayer

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DANKSAGUNG

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ABSTRACT

Biopharmaceutical proteins are mainly produced using recombinant cell technology. During cell line development, a huge number of clones is screened in order to select the best clone suitable for large scale production. Problems concerning instabilities in growth and productivity often lead to a delay and remarkably higher costs in the production processes.

In the framework of this thesis, the commonly used serum-based protocol for cell line generation (including an adaptation step to serum-free conditions that might result to decreased growth rates and productivities) was optimised. We could successfully demonstrate that the basic requirements for transfection of protein-free adapted host cells, namely comparable production rates as well as product qualities, are achievable by our protein-free transfection and screening program. These serum-independently generated clones might be the better model for early investigation of parameters allowing the prediction of the suitability of best producing clones. Further efforts have been undertaken in order to study genetic parameters and their use as additional criteria for clone selection. The method of quantitative PCR to determine gene copy numbers as well as transcript levels was applied systematically during cell line generation. An influence of transcript copy number on specific productivity was clearly evident, whereas a high GCN not necessarily resulted in a high amount of product. Based on our data gained from a typical example of recombinant cell line development, discussion of genetic parameters together with productivity is inevitable in order to get information about bottlenecks during transcription, translation and secretion. Screening for the most balanced clone during selection for subcloning, amplification and fermentation might reduce the potential risk of a loss in productivity and instabilities during the scale-up process. To investigate the insertion loci, the method of fluorescence in situ hybridisation (FISH) was applied to a broad range of recombinant CHO cell lines and resulted in different integration sites enabling stable and high productivities. A slight preference of larger chromosomes as targets for integration was detected.

Due to the development of the protein-free transfection protocol and the platform for analysis of genetic parameters a significant improvement in cell line development could be made.

KURZFASSUNG

Biopharmazeutische Proteine werden überwiegend mit Hilfe der rekombinanten Zelltechnologie hergestellt. Zur Bestimmung des optimalsten Klons für die Produktion im großen Maßstab muss eine Vielzahl an Klonen während der Zelllinienentwicklung gescreent werden. Zusätzlich führen Instabilitäten der Klone hinsichtlich Wachstum und Produktivität zu Verzögerungen und Kostenexplosionen im Produktionsprozess.

Im Rahmen dieser Dissertation wurde das bisherige, auf Serum basierende Verfahren zur Herstellung von Zelllinien – ein Verlust in Wachstum und Produktivität war oftmals eine Folge der Adaptierung an serumfreie Bedingungen – optimiert. Die Hauptanforderungen, nämlich vergleichbare Produktionsraten und Produktqualitäten, konnten mittels unseres proteinfreien Transfektions- und Screeningprogramms erzielt werden. Die von Serum unabhängig erzeugten Klone stellen somit ein weit besseres Modell zur frühen Untersuchung von Parametern dar, die zur Bestimmung der Eignung eines Klons als Produktionsklons erforderlich sind. Ein weiterer Schwerpunkt dieser Arbeit lag in der Analyse genetischer Parameter und deren Einsatz als zusätzliche Kriterien in der Klonauswahl. Die Methode der quantitativen PCR zur Bestimmung von Gen- und mRNA-Kopienzahlen wurde systematisch während der Zelllinienherstellung angewendet. Während ein Einfluss der Transkript-Menge auf die spezifische Produktivität erkennbar war, führen höhere Genkopienzahlen nicht immer zu einer höheren Produktivität. Die Untersuchung der genetischen Parameter in Verbindung mit der Produktivität einer Zelllinie ist daher unverzichtbar, um Engpässe in Transkription, Translation und Sekretion festzustellen. Das Risiko eines Produktivitätsabfalls sowie von Instabilitäten während der Anpassung an die Produktion im großen Maßstab könnte durch die Auswahl eines, im Hinblick auf die genetischen Parameter und Produktivität ausgewogenen Klons für Subklonierung, Amplifikation und Fermentation reduziert werden. Mittels FISH (Fluorescence in-situ hybridisation) wurde die Insertionsstelle in einer Vielzahl von rekombinanten Zelllinien untersucht. In Zelllinien, die eine hohe und stabile Produktivität aufwiesen, wurden verschiedenste Integrationsstellen ermittelt, wobei generell eine Tendenz zur Integration des Fremdgens in größere Chromosomen festzustellen war.

Durch die Entwicklung dieses proteinfreien Transfektionsprotokolls und dem Aufbau einer Plattform zur Analyse von genetischen Parametern konnte somit eine deutliche Verbesserung in der Zelllinienherstellung erzielt werden.

KEYWORDS

recombinant

CHO

protein-free

genetic parameters

screening

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INTRODUCTION

Biopharmaceutical Products

Production of recombinant proteins, e.g. human insulin, growth factor and interferon gamma started in 1978, four years later the first recombinant biopharmaceutical product was marketed. At current, about 500 biopharmaceutical products are supposed to be in the development pipeline leading to estimated sales of about 45 billions of US$ in 2010. The most important product groups of biopharmaceutical proteins involve:

- Blood factors: Factor VIII (hemophelia A) and Factor IX (hemophelia B)
- Thrombolytic agents (e.g. tissue plasminogen activator)
- Hormones like insulin, growth hormone, gonadotropins, follicle stimulating hormone
- Haematopoietic growth factors (e.g. erythropoietin, colony stimulating factors)
- Immune modulators: interferones and interleukines
- Vaccines (e.g. rabies, FSME, influenza vaccine)
- Monoclonal antibodies

In order to remain competitive in the production of recombinant proteins the process has to be optimised and development timelines have to be shortened - in parallel the risks of instabilities in protein production have to be reduced.

The ACBT (Austrian Center of Biopharmaceutical Technology)

The Austrian Center of Biopharmaceutical Technology was founded in September 2001 as a Biotechnology Competence Centre. Improving the efficiency of biopharmaceutical process development in order to enable the fast implementation of optimised production processes for the manufacturing of biopharmaceuticals is the main objective of the ACBT competence centre. The area *Eukaryotic Expression Systems* consists of Sandoz/Tyrol and Polymun/Vienna as industrial and the University of Natural Resources and Applied Life Sciences as academic partner. The work is focussed

on the optimisation of manufacturing processes for post-translationally modified proteins expressed in mammalian cells and on the establishment of an optimised technological platform for the CHO expression system. Our studies "from gene to product" were performed on a Chinese Hamster Ovary (CHO) cell line expressing the model fusion protein EpoFc (see Figure 1) including the following tasks:

- Development of a completely protein independent CHO expression system for the production of recombinant glycoproteins.
- Establishing a platform for analysis of genetic parameters enabling early clone screening
- Identification of important parameters for process optimisation.
- Establishment of tools for analysis of the glycans of complex recombinant glycoproteins enabling early product screening
- Implementation of an interspecies approach to analyse transcription profiles of CHO cells using microarray technology.

The EpoFc Fusion Protein

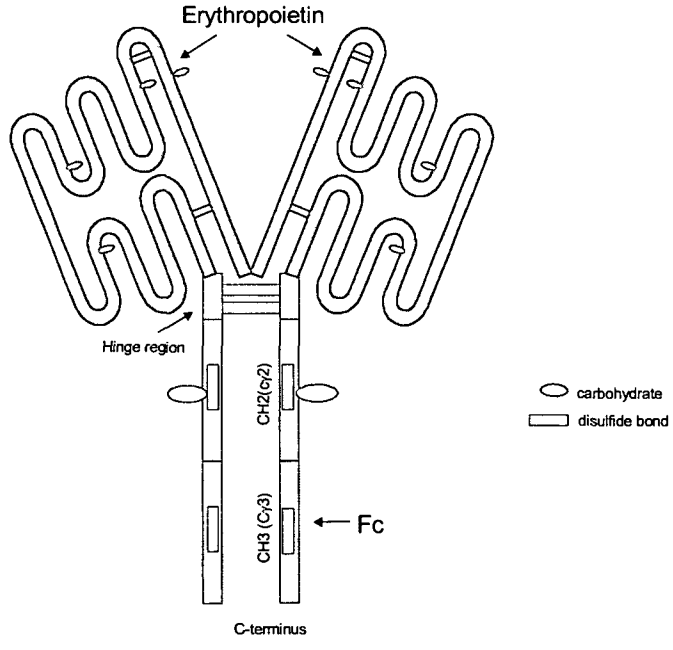

Figure 1: The model fusion protein EpoFc (estimated molecular weight: 112000 Da)

The reason for choosing this fusion protein as model for our studies lies in the advantages conferred by the Fc region:

- Ease of purification: Fc-fusion proteins can be purified in a single-step using protein A or protein G affinity chromatography. Protein A and protein G bind specifically to the Fc region of IgG with affinities that vary accordingly to the IgG isotype.
- Increased half-life in the circulatory system: the Fc region binds to the neonatal constant region fragment (Fc) receptor FcRn resulting in protection from lysosomal degradation. Furthermore Bitoni et al (2002) demonstrated increased half life of EpoFc after delivery to the central airways by using the FcRn transcytosis pathway.

Treatment with erythropoietin – Fc fusion proteins has already been successfully reported (Bitoni et al, 2002 and 2004) and corresponding patents are available:

The invention claimed in US2005202538 provides novel highly sialylated Fc-EPO fusion proteins preferably comprising a couple of modifications in the Fc- portion as well as in the EPO portion and improved pharmacokinetics leading to a prolonged serum half-life and increased in vivo potency. Furthermore the Fc-EPO fusion proteins synthesized in BHK cells have dramatically prolonged serum half-lives and increased in vivo potency when compared to corresponding Fc-EPO fusion proteins produced in other cell lines, such as, for example, NS/O cells.

CN1521192 claims the invention of HuEPO-L-vFc consisting of human epo, a flexible peptide joint with 20 or less amino acids and a human IgG Fc variant leading to a prolonged serum half-time, increased bioactivity and improved dynamic performance and effect of the medicine.

OBJECTIVES

The focus of the present PhD thesis will target on the development of a protein independent CHO expression system and the establishment of a platform for analysis of genetic parameters. After a summary of the molecularbiological as well as biotechnological backgrounds of the research areas of my thesis, the results of our studies will be presented in three publications. In addition, selected chapters of unpublished work are included.

The development and optimisation of a protein-free transfection protocol based on nucleofection for CHO dhfr- grown in suspension as well as the comparison of the obtained clones to recombinant clones derived from a serum-dependent host cell line will be presented in the first publication:

Lattenmayer C, Loeschel M, Schriebl K, Sterovsky T, Trummer E, Vorauer-Uhl K, Muller D, Katinger H, Kunert R. 2006. Protein-free transfection of CHO host cells with an IgG Fusion protein-selection and characterisation of stable high producers and comparison to conventionally transfected clones. Biotechnol Bioeng 26:26.

For the determination of genetic parameters (gene copy numbers and mRNA-levels) the method of quantitative PCR (qPCR) was applied systematically – the results were compared with parameters determined by Southern and Northern blot and can be found in the following study:

Lattenmayer C, Trummer E, Schriebl K, Vorauer-Uhl K, Mueller D, Katinger H, Kunert R 2006. Characterisation of Recombinant CHO Cell Lines by Investigation of Protein Productivities and Genetic Parameters. Biotechnol Bioeng. 2006 Sep 26, doi 10.1002/bit.21183

In order to get a better insight into the genomic level, the insertion sites of the exogenous genes were determined using the method of fluorescence in-situ hybridisation (FISH) in connection with Giemsa-Trypsin banding. For a more detailed investigation of potential integration sites that favour high stability and productivity also other recombinant CHO cell lines were analysed. These investigations are summarised in

Lattenmayer C, Loeschel M, Steinfellner W, Trummer E, Mueller D, Schriebl K, Vorauer-Uhl K, Katinger H, Kunert R. 2006. Identification of transgene integration loci of different highly expressing recombinant CHO cell lines by FISH. Cytotechnology 51(3):171-182.

Further studies of our newly developed recombinant production clones performed in the framework of the ACBT involve process optimisation:

Trummer E, Fauland K, Seidinger S, Schriebl K, Lattenmayer C, Kunert R, Vorauer-Uhl K, Weik R, Borth N, Katinger H, and Mueller D 2006. Process parameter shifting: Part I. Effect of DOT, pH, and temperature on the performance of EpoFc expressing CHO cells cultivated in controlled batch bioreactors. Biotechnol Bioeng. 94(6), 1033-44.

Trummer E, Fauland K, Seidinger S, Schriebl K, Lattenmayer C, Kunert R, Vorauer-Uhl K, Weik R, Borth N, Katinger H, and Mueller D 2006. Process parameter shifting: Part II. Biphasic cultivation-A tool for enhancing the volumetric productivity of batch processes using EpoFc expressing CHO cells. Biotechnol Bioeng. 94(6), 1045-52.

Special care was taken regarding the investigation of the product quality, which is also an important criterion during clone selection:

Schriebl K, Trummer E, Lattenmayer C, Weik R, Kunert R, Mueller D, Katinger H, Vorauer-Uhl K 2006a. Biochemical characterization of rhEpoFc fusion protein expressed in CHO cells. Protein Expr Purif 15, 15.

Schriebl K, Trummer E, Weik R, Mueller D, Kunert R, Lattenmayer C, Katinger H, Vorauer-Uhl K 2006b. A novel strategy for quantitative isoform detection directly performed from culture supernatant. J Pharm Biomed Anal 7, 7.

Beside our investigations that are presented in the publications listed above, the unpublished work on comparison of two different quantitative PCR methods will be presented within this thesis.

FROM GENE TO PRODUCT

Animal Cell Culture Technology

Animal cell culture has become one of the major tools used in cellular and molecular biology. The most important areas where animal cell culture is currently playing a major role will be briefly discussed below:

Model Systems

Animal cell cultures provide a good model system for studying

- basic cell biology and biochemistry
- the interactions between disease-causing agents and cells
- the effects of drugs on cells
- the process and triggers for aging
- nutritional studies

Toxicity Testing

Cultured cells are widely used alone or in conjunction with animal tests to study the effects of new drugs, cosmetics and chemicals on survival and growth in a wide variety of cell types.

Cancer Research

Since both normal cells and cancer cells can be grown in culture, the basic differences between them can be closely studied. In addition, it is possible, by the use of chemicals, viruses and radiation, to convert normal cultured cells to cancer causing cells. Thus, the mechanisms responsible for changes can be studied. Cultured cancer cells also serve as a test system to determine suitable drugs and methods for selectively destroying types of cancer.

Virology

Cell cultures are also widely used in the clinical detection and isolation of viruses, as well as for studies on the mechanisms of viral infections.

Genetic Counselling

Amniocentesis, a diagnostic technique that enables doctors to remove and culture fetal cells from pregnant women, has given doctors an important tool for the early diagnosis of fetal disorders. These cells can then be examined for abnormalities in their chromosomes and genes using karyotyping, chromosome painting and other molecular techniques.

Genetic Engineering

By transfection of cultured cells with exogenous genes molecular biologists are able to study the cellular effects of the expression of theses genes.

Gene Therapy

The ability to genetically engineer cells has also led to their use for gene therapy. Cells can be removed from a patient lacking a functional gene and the missing or damaged gene can then be replaced. The cells can be grown for a while in culture and then replaced into the patient. An alternative approach is to place the missing gene into a viral vector and then "infect'' the patient with the virus in the hope that the missing gene will then be expressed in the patient's cells.

Many gene therapy clinical trials rely on retroviruses, other viruses used as vectors include adenoviruses, adeno-associated viruses, lentiviruses, poxviruses and herpes viruses. Scientists alter these viruses by inactivating genes responsible for reproduction or causing diseases to make them safe for humans and increase their ability to deliver specific genes to a patient's cells

Nevertheless, gene therapy bears several risks: As viruses are usually able to infect more than one type of cell, they might also infect healthy cells. Furthermore, the new gene might be inserted in the wrong location in the DNA, possibly causing harmful mutations to the DNA or even cancer. In addition, when viruses or liposomes are used to deliver DNA to cells inside the patient's body, there is a slight chance that this DNA could unintentionally be introduced into the patient's reproductive cells resulting in changes in the patient's progeny.

Other concerns include the possibility that transferred genes could be "overexpressed," producing so much of the missing protein as to be harmful; that the viral vector could cause inflammation or an

immune reaction; and that the virus could be transmitted from the patient to other individuals or into the environment.

In 2000, gene therapy suffered from a severe setback due to the death of Jesse Gelsinger, an 18-year old teenager, who suffered from ornithine transcarbamylase (OTC) disorder, a rare liver disease,. Gelsinger was injected with adenoviruses. Less than 24 hours after injection, the patient's entire body had been reacting adversely. He went into a coma died two days later.

Drug Screening and Development

Cell-based assays have become increasingly important for the pharmaceutical industry, not just for cytotoxicity testing but also for high throughput screening of compounds that may have potential use as drugs.

Cell-Based Manufacturing

The most important use of animal cell culture is the manufacturing of viruses, proteins and antibodies as well as the use of animal cells as products to replace tissues and organs.

- Production of viruses for use in vaccines: These include vaccines against measles, mumps produced in chicken embryo tissue culture, against poliomyelitis either produced in MRC-5 (a human diploid cell line) or Vero cells, vaccines against rubella and rabies (duck embryo or MRC-5), an attenuated influenza vaccine developed in MDCK or Vero cells, FSME vaccines produced in chick embryo cells and vaccines against Hepatitis A and Varicella produced in MRC-5.
- Protein production: At present, the majority of therapeutic biopharmaceuticals has been produced using animal cell technology and include proteins used for the treatment of cardiovascular diseases (tissue plasminogen activator: tPA), cystic fibrosis (DNases), anemia (erythropoietin), haemophilia (coagulation factors VIII and IX), cancer and viral infections (interferons and interleukins), multiple sclerosis (interferon-beta2) and dwarfism (human growth hormone: hGH).
- Monoclonal antibodies are used for the treatment of a rapidly expanding list of diseases, e.g. to reduce the rejection of transplanted organs by the recipient (anti-CD25: Simulect and anti-TAC: Zenapax), to treat metastasized breast cancer (anti-HER2: Herceptin), to treat non-Hodgkin lymphoma's (anti-CD20: Rituxan), to inhibit allergic asthma (anti-IgE: Xolair), and many others involved in the treatment of inflammation, rheumatoid arthritis and other

diseases. These different antibody indications have in common that effective patient dosing requires relatively large amounts of antibody-protein, which translates into a huge demand for large scale manufacturing capacity. Although only 10% of all biopharmaceuticals relate to monoclonal antibodies, more than 75% of the world wide manufacturing capacity is dedicated to its production.

- Replacement tissues and organs: The first cell-based cartilage product (Carticel) was approved by the FDA in 1995. The treatment involves the cultivation of allogeneic cells in vitro followed by injection into knee defects. In 1997/98, the FDA approved the first human skin products (Apligraf and TransCyte) consisting of human dermal fibroblasts seeded onto collagen matrices or synthetic polymer scaffolds to treat venous ulcers as well as full- and partial-thickness burns. Further investigations involve artificial organs such as pancreas, liver and kidney and the creation of entirely biologic vascular structures. A potential supply of replacement cells and tissues may come out of work currently being done with both embryonic and adult stem cells.

Host Cell Lines

Mammalian cells are commonly used as heterologous expression systems as they allow correct protein folding to tertiary structures as well as posttranslational modifications that are necessary for the therapeutic activity of many proteins (Barnes et al, 2002). Among the mammalian cells (see Table 1) used for production of proteins the following cell lines are of particular commercial importance:

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

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

[b]E-mail: *lac2@columbia.edu*

Table 1: Common mammalian host cells (Gray, 1997)

Most biopharmaceuticals on the market are produced in immortalised **Chinese Hamster Ovary (CHO)**-derived cell lines, which have been used extensively for high-level protein production because they enable amplification of specific genes through selection with an amplifiable marker such as dihydrofolate reductase (dhfr) or glutamine synthetase (GS).

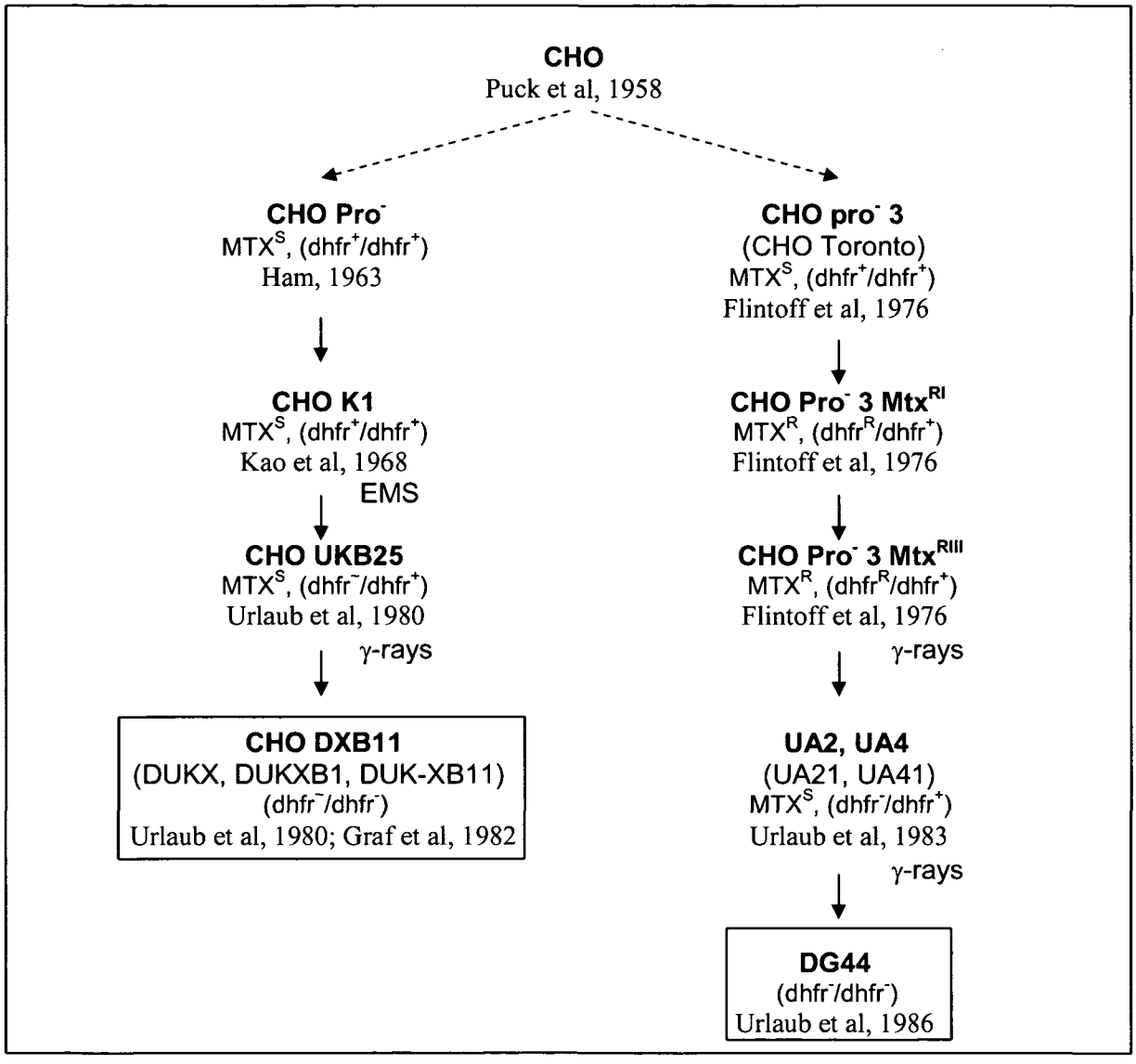

Figure 2: History of the CHO cell lines

The history of the CHO cell lines (see Figure 2) started in the 1950's, when Puck et al (1958) investigated the growth properties and karyotypes of cell cultures derived from biopsies from lung, kidney and ovaries from a partially inbred female adult Chinese hamster (*Criteculus griseus*) and other rodents. The hamster cells were discovered to be most interesting for genetic studies due to their rather low chromosome number (2n=22; Tjio et al, 1958). As cells derived from an ovary biopsy did not

change in growth, morphology and karyotype during continuous cultivation over more than 10 months, these cells were regarded as particularly hardy and reliable. Their growth behaviour was described as "fibroblast-like" as the cell grew as elongated structures tending to align in parallel fashion to form colonies with rough edges. A defined medium for single cell plating was developed by Ham (1962). One subclone CHD-3A derived from a chinese hamster ovary which had been routinely cultivated for several years was subsequently found to require proline for growth (Ham, 1963) due to the lack of the enzyme converting glutamic acid to γ-semialdehyde and contained in contrast to the initial cell line only 21 chromosomes (Ham, 1965; Kao et al, 1967). CHO pro$^-$ K1 (also known as CHO K1) was a subclone derived from the proline-deficient cell line and exhibited a chromosome number of 20 (Kao et al, 1968). Further studies were performed by Kao et al (1968) using the 5-bromodeoxyuridine (BUdR) visible light technique. The initial cell line as well as CHO pro$^-$ K1 were able to grow in F12 medium (Ham, 1965) lacking glycine, alanine, aspartic acid, glutamic acid, thymidine, hypoxanthine, inositol, vitamine B12 and lipoic acid. After mutagenesis of the K1 subclone by ethylmethanesulfonate (EMS) and N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) auxotrophic mutants requiring glycine, thymidine and hypoxanthine were isolated by the BUdR technique that destroys prototrophs by exposure to near-visible light. Due to the metabolic pathways of these components the mutants were supposed to represent deficiencies in dihydrofolate reductase (dhfr; for metabolic pathways see Figure 5 and 6). The central role of this enzyme in the synthesis of nucleic acid precursors together with its great sensitivity to tetrahydrofolate analogues such as Methotrexate (MTX, also known as amethopterin) has made this enzyme a target of wide use in cancer therapy (Bertino, 1979) and led to further investigations on the deletion of the dhfr locus. Urlaub et al (1980) isolated mutants of CHO pro$^-$ K1 derivated cells lacking dhfr activity after two discrete mutagenic steps. The first step yielded a heterozygote containing one wild-type and one mutant (non-functional) allele after mutagenesis with EMS and selection with [^{3}H]-deoxyuridine (dUrd) in the presence of MTX (e.g. the clone UKB25 was obtained after this step). Mutants fully deficient in dhfr (and thus requiring glycine, a purine and Thymidine for growth) were obtained in a second step, when the heterozygotes were selected again with [^{3}H]-dUrd but in the absence of MTX after mutagenesis with EMS or γ-rays. The commonly used DXB11 was obtained by the latter method (Graf et al, 1982).

Other synonyms of this cell line are DUKX, DUKXB1, DUK- or DUK-XB11. Inactivation of at least one of the dhfr genes was not irreversible (Gandor et al, 1995) – most likely the allele already inactivated in UKB25 as it was isolated after mutagenesis with EMS, a base substitution mutagen. In contrast, the second allele was inactivated by gamma radiation that was more likely to have induced an irreversible deletion. As CHO DXB11 is the most widely used dhfr mutant, it is important to be aware of the possibility of reactivation of the dhfr gene.

Another famous and commonly used CHO cell line is DG44. Its history started in 1976, when Flintoff et al investigated the characteristics of the MTX system in somatic cells. They selected a highly resistant cell line (CHO Pro$^-$ 3 Mtx RIII) derived from the CHO Pro$^-$ 3 subclone of the CHO cell line established in the 1950s (also known as CHO Toronto because it was used extensively in that city; Gray, 1997) in two steps: The first step yielded a mutant that produced wild-type levels of an altered dhfr enzyme binding MTX with a lower affinity and therefore leading to resistance to lower MTX concentrations. In a second step a mutant resistant to higher levels of MTX due to dhfr gene amplification was selected.

Urlaub et al (1983) were convinced that a hemizygous mutant (a mutant in which one allele has been physically eliminated by deletion) would be of greater benefit for genetic analysis of mutations in the wild-type dhfr than the heterozygous DXB11 clone they had developed a few years earlier. Therefore the CHO cell line Pro$^-$ 3 Mtx RIII generated by Flintoff et al (1976) with one allele containing 20 copies of dhfr with a higher resistance to MTX was subjected to mutagenesis. The reason for choosing this cell line was the differential MTX sensitivity of the wild-type versus the altered enzyme expressed in Pro$^-$ 3 Mtx RIII which allowed, during selection, the use of MTX concentrations that inactivate the wild-type dhfr without substantially inhibiting the altered enzyme. Pro$^-$ 3 Mtx RIII were therefore exposed to gamma irradiation as a mutagen, since this treatment had been shown to be capable of causing cross DNA sequence changes at the dhfr locus in CHO cells (Graf et al, 1982). After mutagenic treatment and several selection steps in MTX and [^{3}H]-dUrd, the clones UA2 and UA4 were obtained with dfhr activities indistinguishable from the wild-type clone with respect to MTX sensitivity due to deletion of the amplified mutant dhfr allele with a higher MTX resistance. In order to

investigate whether γ-irradiation was an efficient mutagen for the induction of major changes in the gene structure of mammalian cells, efforts have been undertaken in order to delete the wild-type allele that was still present and active in UA2 and UA4 (Urlaub et al, 1986). After γ-irradiation and selection of dhfr-deficient mutants, finally the cell line CHO DG44 was obtained, with a deletion of at least 115 kb. Beside CHO DXB11 ($dhfr^+/dhfr^-$), DG44 ($dhfr^-/dhfr^-$) is the most widely used cell line in combination with the dhfr selection system.

All of the **Murine myeloma cells** used commercially today originate from the plasmacytoma tumour MOPC21 (Shulman et al., 1978) induced in a mouse (*Mus musculus*) by peritoneal injection of mineral oil. Myeloma cells from this tumor were then cloned and selected until the nonsecreting cell line, **NS0**, was obtained (Barnes et al, 2000), which is most often used in combination with the glutamine synthetase (GS) selection system (patented by Lonza) due to the GS deficiency. Another cell line derived from this tumor is **Sp2/0**, which is mainly used for recombinant antibody production, and is also a commonly used fusion partner for generation of hybridoma cells.

Hybridoma cell lines are generated by fusion of an antibody-producing B lymphocyte and a lymphocyte tumor (or myeloma) cell from pristane-oil induced cancerous B lymphocytes in Balb/c mice (Milstein, 1980). Therefore they possess the property of infinite life due to their tumorigenic background and have the machinery to produce large amounts of antibodies making hybridoma cell lines to commercially relevant sources of monoclonal antibodies.

The difficulty is selecting for the hybridomas, since the majority of cells after the fusion are myeloma cells that did not fuse. The solution to this problem was to exploit purine and pyrimidine salvage pathways in a selection scheme. Koehler and Milstein used myeloma cells that were unable to use the purine salvage pathway because they were defective in HGPRT (hypoxanthine guanine phosphoribosyl transferase) due to the treatment with the hypoxanthine analogues 6-thioguanine or 8-azaguanine – as an alternative thymidine kinase (TK; obtained after exposure to the thymidine analogue bromodeoxyuridine) deficient cells can be used. As HGPRT and TK reside on the X chromosome, a single mutation is sufficient for generating HGPRT- or TK- phenotypes. The

predominantly used myeloma cell lines (Sp2/0, NS1, NSO, and X63Ag8) were preselected for HGPRT deficiency.

In the cells, two pathways of nucleotide biosynthesis exist (see Figure 3): the de novo pathway, which requires glutamine and aspartate respectively (as well as activated phosphate) as initial substrates for a series of reactions for the synthesis of purine-type (DATP and dGTP) and pyrimidine-type (dCTP and dTTP) dNTPs, and the salvage pathway, which uses HGPRT for generation of IMP from hypoxanthine and TK to convert thymidine to dTMP.

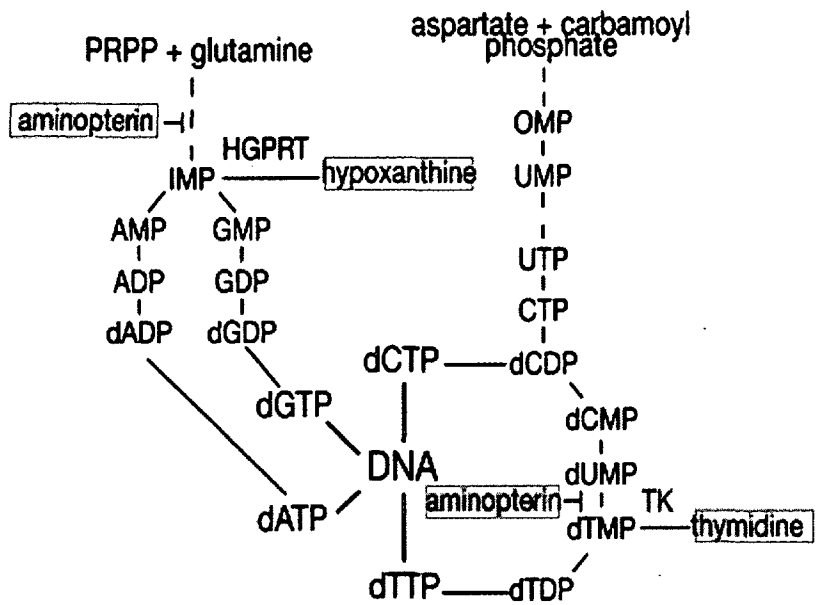

Figure 3: Pathways involved in the synthesis of nucleotides in mammalian cells (http://nfs.unipv.it/nfs/minf/dispense/immunology/monab.html)

Several of the reactions involved in the de novo pathway of dNTPs can be blocked by aminopterin, an analogue of dihydrofolate (another one is Methotrexate also known as amethopterin) that binds with very high affinity and blocks the enzyme dihydrofolate reductase. Whereas the B lymphocytes survive in Littlefield's HAT medium containing aminopterin, hypoxanthine and thymidine (as they can utilise the salvage pathways) the myeloma cells either deficient in HGPRT or TK will die. The immortal hybrid survives due to utilisation of the normal HGPRT or TK gene of the B cell and outgrows the B lymphocytes, the unfused myeloma cells die.

Baby hamster kidney (**BHK,** derived from the kidney cells of baby Syrian golden hamsters (*Mesocricetus auratus*)) and human embryo kidney (**HEK-293,** an epithelial cell line derived from

human embryonic kidney cells transformed with adenovirus 5 DNA) are further cell lines that have gained regulatory approval for recombinant protein production.

A rather new cell line on the market, **PER.C6**, is currently under evaluation by several biopharmaceutical companies and was made by immortalizing healthy human embryonic retina cells with an adeovirus E1 gene sequence (Fallaux et al, 1998; Jones et al, 2003). The disadvantage of this trademarked cell line (US2006063234) mainly lies in its viral background, although no adventitious viruses and retroviruses were detected and a Biologics Master File is held at the FDA (Food and Drug Association). Its main advantage is the human origin as well as the ability to grow to comparable high cell densities. It requires no amplification of inserted genes to deliver stable clones with high levels of protein expression in several months, nor does it require any selection agent. A low copy number is sufficient to retain stability and efficient protein expression.

The cell line we used for our studies was CHO/dhfr- [CHO DUK-, another synonym for CHO DXB11] obtained from the American Type Culture Collection (ATCC; Catalog Number CRL-9096 deposited from Hoffmann-La Roche). According to the ATCC, the cell line is cited in a U.S. and/or other Patent or Patent Application, and may not be used to infringe on the patent claims. Nevertheless, an infringement search only revealed that the cell line CRL-9096 isolated by Urlaub et al (1980) is cited, but not claimed in US Patent 4992367 from Hoffmann-La Roche. Therefore the use of this cell line is not restricted.

Transfection

A variety of methods is available for incorporation of foreign DNA into mammalian cells. The challenge of transfection is to introduce negatively charged molecules (e.g., phosphate backbones of DNA and RNA) into cells with a negatively charged membrane. Chemicals like calcium phosphate and DEAE-dextran or cationic lipid-based reagents coat the DNA, neutralizing or even creating an overall positive charge to the molecule. Physical methods like microinjection or electroporation simply punch through the membrane and introduce the DNA directly into the cytoplasm.

Chemical Methods

- Coprecipitation with Calcium-Phosphate is widely used as the components are easily available and inexpensive. Furthermore the protocol is easy-to-use and can be applied to different cultured cell types. Coprecipitation of DNA with calcium-phosphate (Graham et al, 1973; Wigler et al, 1979) is achieved after mixing DNA with calcium chloride in a buffered saline/phosphate solution. The precipitate is then adsorbed onto the cells, and finally enters the cells via endocytosis or phagocytosis. Calcium phosphate transfection is routinely used for both transient and stable transfection of a variety of cell types. To increase efficiency of transfection for some cell types, additional treatments such as glycerol, dimethyl sulfoxide chloroquine and sodium butyrate may be added during incubation with the calcium phosphate/DNA precipitate. These treatments are thought to disrupt the phagocytic vacuole membrane, allowing the DNA to be released to the cytoplasm (Felgner, 1990).

- Dimethylaminoethyldextran (DEAE-Dextran) is one of the first chemical reagents used for transfer of nucleic acids into cultured mammalian cells (McCutchan et al, 1968). The cationic polymer DEAE-Dextran tightly associates with negatively charged nucleic acids. An excess of positive charge, contributed by the polymer in the complex, allows the complex to come into closer association with the negatively charged cell membrane. Uptake of the complex is presumably by endocytosis. Transfection efficiencies can be increased in many cell types by additional treatments after the primary exposure of the cells to DEAE-dextran and DNA. The

most effective and routinely used agents are glycerol, dimethyl sulfoxide (DMSO) and chloroquine. This method is successful for delivery of nucleic acids into cells for transient expression that leads to short-term expression of a few days in duration (Gluzman, 1981).

Other synthetic cationic polymers have been used for the transfer of DNA into cells, including polybrene (Kawai et al, 1984), polyethyleneimine (Boussif et al, 1995) and dendrimers (Haensler et al, 1993; Kukowska-Latallo et al, 1996).

- Use of Cationic Lipids: Fraley et al (1980) used artificial liposomes to deliver DNA into cells. Further advances were achieved by Felgner et al (1987) when they developed synthetic cationic lipids: The cationic portion of the lipid molecule associates with the negatively charged nucleic acids, resulting in compaction of the nucleic acid in a liposome/nucleic acid complex, presumably from electrostatic interactions between the negatively charged nucleic acid and the positively charged head group of the synthetic lipid. Entry of the liposome complex into the cell may occur by the processes of endocytosis or fusion with the plasma membrane via the lipid moieties of the liposome (Gao et al, 1995). Following cellular internalization, the complexes appear in the endosomes and later in the nucleus. Often the cationic lipid is mixed with a neutral lipid such as L-dioleoyl phosphatidylethanolamine (DOPE), which is considered a "fusogenic" lipid (Farhood et al, 1995), and its role may be to release these complexes from the endosomes as well as to facilitate fusion of the lipid bilayer of the outer cell membrane with the liposome/nucleic acid complexes. Liposome-mediated delivery offers advantages such as relatively high efficiency of gene transfer, successful delivery of DNA of all sizes from oligonucleotides to yeast artificial chromosomes, delivery of RNA and protein and furthermore allows stable transfection that rely upon integration of the DNA into the chromosome. Unlike the other chemical methods described here, liposome-mediated nucleic acid delivery (lipofection) can be used for in vivo transfer of DNA and RNA to animals and humans (Felgner et al, 1995).

  A suitable cationic lipid reagent is Lipofectamine (Invitrogen, US Patent 5334761), that we used for transfection of our serum-dependent CHO cell line. Lipofectamine is a 3:1 (w/w)

liposome formulation of the polycationic lipid 2,3-dioleyloxy-N-[2(spermine-carboxamido)-ethyl]-N,N-dimethyl-1-propana-minium trifluoroacetate (DOSPA), and the neutral lipid DOPE.

Physical Methods

- Electroporation was first reported for gene transfer studies into mouse cells (Wong et al, 1982) and is the exposure of a cell suspension to a high voltage electrical impulse (100-200 V for 1-2 ms) resulting in transient pores which enable uptake of the DNA (Shigekawa et al, 1988).

  The technique requires fine-tuning and optimization for duration and strength of the pulse for each type of cell used. In addition, electroporation often requires more cells than chemical methods because of substantial cell death, and extensive optimization is often required to delicately balance transfection efficiency against cell viability.

  In our studies, electroporation of serum-independent host cell was performed using the Nucleofector™ technology (US Patent 2004014220) from Amaxa which allows - due to the use of cell-line dependent parameters (electroporation buffer composition, voltage, pulse length) – direct integration of the foreign DNA into the nucleus. Especially the very high field strengths of 2 to 10 kV/cm are used to aid DNA in entering the nucleus independently of cell division. These field strengths are substantially higher than the ones generally used in electroporation, and are also higher than the field strengths that are sufficient for efficient opening of cell membrane pores. The high voltages may result in the generation of pores in both membranes of the nuclear envelope, or the nuclear pore complexes may become more permeable for molecules, thus enabling very efficient transport of the biologically active molecules into the nucleus.

- By Microinjection the DNA is inserted by a micro-needle into the nucleus of individual cells (Cappechi, 1980). However, the apparatus is costly and the technique extremely labour-intensive, thus it is not an appropriate method for studies that require a large number of transfected cells.

- Another physical method of gene delivery is Biolistic Particle Delivery, also known as Particle Bombardment. This rather costly method relies upon high velocity delivery of nucleic acids on microprojectiles to recipient cells by membrane penetration (Ye et al, 1990). This method has been successfully employed to deliver nucleic acid to cultured cells as well as to cells in vivo.

Biological Methods

- Protoplast Fusion: Plasmids grown in bacteria can be introduced directly into mammalian cells by cell fusion with the bacterial protoplast, which is obtained by lysozyme treatment of the bacteria in order to remove the cell wall. Fusion is then enabled in the presence of polyethylenglycol (PEG).

- Viral Methods: Animal viruses increase the efficiency of nucleic acid transfer into mammalian cells (a process called transduction) due to the following properties:
    - they promote transfer of DNA because of capsid proteins that bind to cell membrane receptors
    - they contain promoters for gene expression in mammalian cells
    - they replicate to high copy numbers
    - some viruses integrate efficiently into the animal cell genome

  Adenoviral vectors rapidly infect a broad range of human cells, are able to achieve high levels of gene transfer and can accommodate relatively large segments of DNA (up to 7.5kb). Retroviruses that are able to integrate genes into the nuclear DNA (Cepko et al, 1984) and a subset of the retrovirus family, lentiviruses (e.g., HIV-1) are of particular interest because they have been well-studied, can infect quiescent cells, and can integrate into the host cell genome to allow stable, long-term expression of the transgene (Anson, 2004). Vectors derived from a simian virus (SV40; Rigby, 1982) have the advantage of a very efficient transduction by viral infection. However, this system has a limited host range (only monkey cells) and is suitable only for small foreign DNA inserts. Other vectors of interest include the adeno-associated viruses and the herpes simplex viruses.

Media

In the past, serum added at a concentration of 1-20% was essential for the propagation of mammalian cells. Serum from fetal calves (FCS) was used frequently due to its higher concentrations of certain growth factors (for example insulin and growth hormone) than serum from other species (e.g. bovine or human serum). Bovine serum has a typical protein content of 60-80 g/L. Table 2 shows the list of components supplied by serum. Bovine serum albumin (BSA) is the main protein present in serum with a concentration of 30-50 g/L.

- **Growth factors and hormones as mitogens** (e.g. insulin, PDGF, FGF, EGF)
- **Transport proteins and detoxifying** agents (e.g. albumin and transferrin)
- **Attachment factors** (e.g. collagen, fibronectin, vitronectin)
- **Protease inhibitors** (e.g. trypsin inhibitor)
- **Shear protective agents** (e.g. albumin and bulk proteins)
- **Nutrients and energy source** (e.g. glucose)
- **Purines and pyrimidines**
- **Vitamins** (B-group, C and E)
- **Trace elements and inorganic compounds** (e.g. Cu, Sn, Mn, Va, Zn and Se)

Table 2: Components supplied by serum (Keenan et al, 2006)

Recent demands of economy, reproducibility, transportability, availability of qualified raw material and regulatory demands have led to efforts of eliminating serum from cell culture media. Medium prepared with serum at 10% has a protein concentration of 6 to 8 g/L. A typical recombinant protein produced in mammalian cells is anywhere from a few mg/L to 1 g/L. The serum proteins thus become a major contaminant of any crude supernatant in which the target protein accumulates. Further, if the target protein is functionally, biochemically or physically related to a serum protein, then it may prove difficult to separate the target protein from the serum protein.

Nevertheless, the replacement of serum was not a straightforward approach. Its complexity has made it difficult to identify all components necessary for growth of cultured cells.

While supplementation of basal media with additional vitamins, trace elements, glucose and nucleotides (Ham, 1984) reduced one part of the additives required for growth, protein and lipid

components still had to be added in order to obtain so-called serum-free media (SFM). Albumin, hormones and growth factors (e.g. insulin, transferrin, hydrocortisone, FGF, EGF) are added.

The protein concentration of serum-free media is between 50 mg/L and 1 g/L. Unlike serum, the composition is known and typically three proteins, albumin, transferrin and insulin, comprise 80% to 90% of the proteins present. Consequently, the relative level of the target protein is significantly higher and with fewer contaminants. Thus, the target protein is easier to purify in fewer steps which gives higher recovery values. As the proteins are often derived from of animal or human origin, the question of their origin leads to further regulatory demands (e.g. TSE and other adventitious agents). Many serum-free formulations for recombinant CHO cells expressing a variety of proteins were described, but a versatile serum-free medium formulation for the parental CHO DUKXB11 has not been reported to date (Schroeder et al, 2004).

The efforts in developing protein-free (PFM) media often resulted in decreased cell growth and recombinant protein production (Castro et al, 1992; Stoll et al, 1996; Lee et al, 1999). Therefore low-cost hydrolysates containing undefined mixtures of low-molecular weight components, including amino acids, peptides, vitamins and trace elements, are frequently utilised as protein-free media additives to provide nutrients in cell culture (Dyring et al, 1994; Zhang et al, 1994; Nyberg et al, 1999; Jan et al, 1994). Hydrolysates are enzymatic or acidic digests of biological material such as animal tissues, milk products, microorganisms and various plants (e.g. soy, wheat gluten, rice) supplying peptides, amino acids, vitamins and trace elements. For the production of therapeutic proteins, non-animal derived hydrolysates (e.g. hydrolysates derived from yeast or plants) are used (Sung et al, 2004; Schroeder et al, 2004) in so-called animal-derived component free PFM. Hydrolysates were also found to have a beneficial effect on unwanted proteolysis of secreted proteins (Mols et al 2005).

The long-term cooperation of our institute with the Institute of Experimental Botany in Prague focusing on the effects of plant protein hydrolysates and synthetic oligopeptides in animal cell culture media (Franek et al, 2000, 2002 and 2003) has resulted in the development of our protein-free medium that enabled generation of various protein-free adapted recombinant CHO cell lines.

Even more favoured by regulatory authorities are fully chemically defined media (CDM). Significant progress has recently been made in substituting undefined components. As discussed before, the substitution of protein and lipid components of serum is still the major hurdle in developing CDM. In the following section, the most important components of serum and their substitutes, as well as further additives to CDM will be discussed.

Insulin is a common protein supplement found in most serum-free media formulations (Barnes et al, 1980; Jayme, 2000) in a concentration up to 10 mg/L. Insulin acts a mitogen for most cells and is involved in glucose and lipid pathways and influences amino acid uptake and DNA synthesis (Hoffmann et al, 1989). Insulin mimetics may offer a limited alternative replacement for insulin in SFM (Tang et al, 2001; Zhang et al, 2005). In order to avoid regulatory concerns, animal derived insulin was substituted by recombinant insulin produced from *E.Coli*, although this is a relatively costly option. Among the trace elements that have been reported to exhibit insulin-like effects, zinc is the most promising one. Wong et al (2004) demonstrated that substitution of insulin by zinc did not affect cell growth, cell cycle progression and antibody production of CHO-K1. Although the exact mechanisms of zinc leading to substitution of insulin yet remain unclear, transcription profiling indicated no major change in the global expression profile between insulin and zinc supplemented culture (Wong et al, 2006). An alternative strategy was used by Schröder et al (2004). By weaning CHO DUXB11 cells from SFM with reducing insulin concentrations, a protein-free medium was obtained that allowed growth of a recombinant CHO DUXB11 cell line.

The most commonly used chelator of iron in SFM is **transferrin** (Barnes et al, 1980) in a concentration up to 35 mg/L (Butler, 2004). Iron supports various metabolic processes and cell growth. The addition of iron without a carrier can be problematic. In aqueous solution, non protein-bound iron can lead to free radical formation and toxic effects on cells. Furthermore, the formation of insoluble ferric hydroxides can make the iron biologically unavailable (Keenan et al, 2006). A variety of iron chelators were used to replace recombinant transferrin for specific cell lines, but none could support the universal activity of transferrin (Sanders et al, 1988). The ability of simple ferric compounds (ferrous sulphate, ferric chloride, sodium nitroprusside and ferric ammonium sulphate) to

replace transferrin has been reported for a variety of cells (Keenan et al, 1996). Kovar et al (1987) discovered the ability of ferric citrate to replace transferrin over 30 passages. Also more complex synthetic chelators, often protected by a patent, such as tropolone (2-hydroxy-2,4,6-cycloheptatrien-1; Metcalfe et al, 1994) and ferric pyridoxal isonicotinoyl hydrazone (FePIH; Tsao et al, 1987) have been used for certain cell lines. Recently the novel function of selenite as an iron carrier was discovered (Zhang et al, 2006).

Albumin (especially bovine-derived BSA) has been incorporated into a variety of SFM in a concentration between 1-5 g/L (Castro et al, 1995) as a lipid transport system (in order to overcome problems of poor solubility and toxicity of lipids), source of intermediary metabolites and protection from shear- and oxidative stress. Naturally bound ligands of albumin include bilirubin, fatty acids (especially long-chain fatty acids, e.g. linoleic, arachidonic, palmitic, linolenic and oleic acids), vitamins, amino acids, hormones, globulins and metal ions (Keenan et al, 2006). Beside from recombinant albumin, plant derived cyclodextrins (Gorfien et al, 2000; Jayme, 1999; Zhang et al, 2005) and synthetic low density lipoprotein complexes (Hayari et al, 2005) offer an opportunity to replace animal-derived lipid transporters.

Further supplements to CDM include Pluronic F-68 and selenium: **Selenium** is well-known essential trace element that protects cells from oxidative damage. It can either be used as an antioxidant or a pro-oxidant and typically exists in selenoproteins in vivo and in various oxidative states in vitro. The established mechanisms include its activation of glutathione peroxidase, a key enzyme in the defence of oxidative stress and its role as a cofactor of glutathione synthetase that also detoxifies cell damaging free radicals. Garcia-Briones et al (1992) showed that the presence of **Pluronic F-68**, a block copolymers based on ethylene oxide and propylene oxide, prevents cell adhesion leading to decreased cell damage.

In addition, **lipids or lipid precursors** such as choline, linoleic acid, ethanolamine or phosphoethanolamine are often added to CDM.

To conclude, only little information is available concerning CDM as they are often protected by patents. Therefore, not the entire list of substitutes for serum components can be given in the

framework of this thesis. For example, Invitrogen recently developed a chemically defined formulation that contains neither proteins nor hydrolysates of either plant or animal origin. Growth of mammalian cells is facilitated due to the replacement of insulin by zinc and an iron-chelate instead of transferrin. Moreover, supplementation with dextran sulphate not only facilitates growth at high cell densities and increases the expression level of proteins (US2006148074).

In the framework of this thesis the current serum-based transfection protocol of the industrial partners for cell line generation had to be optimised (see Figure 4) in order to obtain a serum-free or, even preferred, a protein-free transfection protocol. Since the cultivation of recombinant cell lines in protein-free media has a long history at the IAM and Polymun we were able to start with a protein-free adapted host cell line CHO DXB11 and developed a protocol for transfection of serum-independent CHO cells that resulted in stable and high producing recombinant production clones.

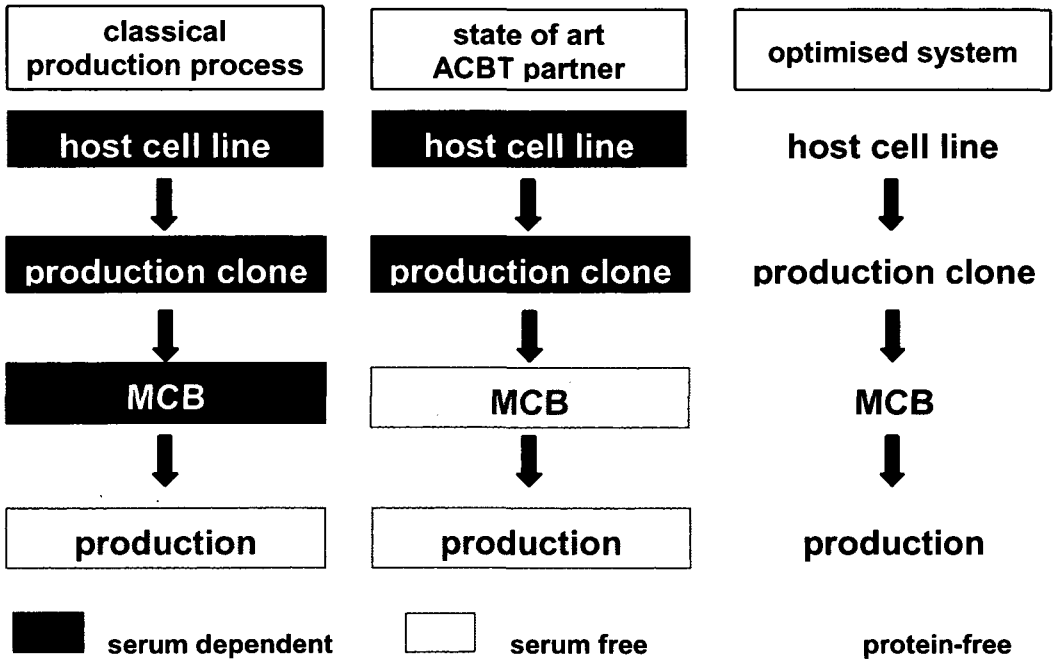

Figure 4: Protocols of the industrial partner of the ACBT for recombinant cell line generation

The basis media we used for our recombinant EpoFc clones during cell line development were Dulbecco's Modified Eagle Medium (DMEM), whereas for production cultivation was performed in a DMEM/Ham's F12 1:1 mixture. Both media were then supplemented with glutamine and FCS for the serum-dependent EpoFc clones, and with glutamine, soy-peptone, Pluronic F-68 and an in-house

developed protein-free supplement in case of the protein-free clones. MTX was added to the media according to the selection step.

	DMEM	DMEM/Ham's F12 1:1
	[mg/L]	[mg/L]
INORGANIC SALTS		
Calcium chloride anhydrous	200,00	116,61
Cupric sulfate pentahydrate		0,0013
Ferric nitrate nonahydrate	0,10	0,05
Ferrous sulfate heptahydrate		0,42
Magnesium chloride anhydrous		28,61
Magnesium sulfate anhydrous	97,67	48,84
Potassium chloride	400,00	311,8
Sodium bicarbonate	3700,00	2438
Sodium chloride	6400,00	6999,5
Sodium phosphate dibasic anhydrous		71,02
Sodium phosphate monobasic monohydrate	125,00	62,5
Zinc sulfate heptahydrate		0,43
VITAMINS		
Biotin		0,0037
Choline chloride	4,00	8,98
Cyanocobalamin		0,68
D-calcium pantothenate	4,00	2,24
Folic acid	4,00	2,65
i-inositol	7,00	12,6
Niacinamide	4,00	2,02
Pyridoxine HCl	4,00	2,03
Riboflavin	0,40	0,22
Thiamine HCl	4,00	2,17
AMINO ACIDS		
Glycine	30,00	18,75
L-alanine		4,46
L-arginine HCl	84,00	147,5
L-asparagine monohydrate		7,5
L-aspartic acid		6,65
L-cysteine HCl monohydrate		17,56
L-cystine 2HCl	62,57	31,29
L-glutamic acid		7,35
L-histidine HCl monohydrate	42,00	31,48
L-isoleucine	104,80	54,47
L-leucine	104,80	59,05
L-lysine HCl	146,20	91,25
L-methionine	30,00	17,24
L-phenylalanine	66,00	35,48
L-proline		17,25
L-serine	42,00	26,25
L-threonine	95,20	53,45
L-tryptophan	16,00	9,02
L-tyrosine 2Na dihydrate	103,79	55,79
L-valine	93,60	52,85
OTHER		
Dextrose anhydrous	individually	individually
DL-α-Lipoic acid		0,105
Hypoxanthine sodium salt		2,39
Linoleic acid		0,042
Phenol red sodium salt	15,93	8,60
Putrescine 2HCl		0,081
Thymidine		0,365

Table 3: Comparison of DMEM to DMEM/Ham´s F12 1:1

When comparing the composition of DMEM to the DMEM/Ham's F12 1:1 mixture (see Table 3), despite the reduced amino acid and vitamin concentrations in the mixture the main difference between the two media is the presence of lipoic and linoleic acid, putrescine as well as hypoxanthine and thymidine in the DMEM/HAM's F12 mixture. As the selection of positive transfectants using the dhfr system (which is discussed in the next chapter) is based on the ability of growing in a medium lacking glycine, hypoxanthine and thymidine, only stable clones should be cultivated in this medium.

Lipoic acid, a water and lipid soluble disulfide fatty acid, participates in numerous chemical processes both inside and outside of the cell and is able to move across the cell membrane of lymphocytes and fibroblasts. It is involved in energy and amino acid metabolism; furthermore its reduced form, dihydrolipoic acid, stimulates glutathione synthesis by improving cystine utilization. Under conditions of oxidative stress, lipoic acid may replace insulin as an agent that supports increased glucose uptake. Especially the antioxidant functions, the capability to repair proteins damaged by oxidation and the protection of cell membranes from lipid peroxidation make lipoic acid together with its reduced form an especially important component of serum- and protein-free media.

Linolenic acid is an unsaturated fatty acid that is not synthesized by animal cells and must be provided as a nutrient in cell culture. Historically, it has been provided to cells in culture as a component of serum, albumin complex or esterified to molecules such as cholesterol. The advent of serum-free, animal-protein-free and protein-free media formulations has increased the difficulty of delivering linolenic acid to cells in culture.

Its primary function is the long-term storage of energy derived from NADPH and ATP. Furthermore, fatty acids like linolenic acid are precursors of other molecules like prostaglandins, prostacyclins, thromboxanes, phospholipids, glycolipids, and vitamins; in addition fatty acids are important constituents of cell structures such as the membranes.

Only little information is available concerning the function of putrescine (1,4-diaminobutane or butanediamine). The polyamines, of which putrescine is one of the simplest, appear to be growth factors necessary for cell division.

Selection Systems and Gene Amplification

In order to allow identification of the cells that have been successfully transfected, a genetic marker can either additionally be integrated into the plasmid carrying the exogenous target or can be cotransfected on a second vector. Gene markers that have been used extensively in mammalian cells include genes for the enzymes hypoxanthine guanine phosphoribosyl transferase (HGPRT), thymidine kinase (TK) and dihydrofolate-reductase (dhfr). These are recessive marker genes and can only be used for selection in a population of cells that are mutants lacking the marker enzyme activity prior to transfection.

Antibiotic resistance genes are dominant markers. For example, the neomycin-resistance gene (neo^R) codes for the enzyme aminoglycoside phosphotransferase, which inactivates the aminoglykoside group of the antibiotics G418, neomycin and kanamycin by phosphorylation. This inactivation is thought to interfere with the active transport of the antibiotics into the cell.

G418 sulphate (Geneticin), a 2-deoxystreptamine antibiotic produced by *Microspora rhodorangea*, is structurally related to the aminoglycoside gentamicin but has inhibitory activity against a greater variety of pro- and eukaryotic organisms. Expression of aminoglycoside phosphotransferase in eukaryotic cells enables transfected cells to grow in media containing G418 sulfate (Hadfield et al, 1990) and thus allows the elimination of nontransformed eukaryotic cells due to binding to ribosomal components and thus inhibiting protein synthesis. In contrast, neomycin mainly affects prokaryotic cells and inhibits the bacterial organelle.

Another dominant marker is glutamine synthetase (GS), which is used in CHO and myeloma cells.

Beside the selection of the positively transfected genes, the dhfr and the GS system can be used for gene amplification in order to increase the low productivity of mammalian cells. The most frequently used cell lines for heterologous expression using the dhfr selection and amplification system are CHO DXB11 and DG44.

To understand dhfr selection, the role of folates in metabolic pathways will be briefly discussed: The folates are used as coenzymes for the oxidation, reduction and transfer of one-carbon units in various

metabolic pathways that lead to the biosynthesis of thymidine, purines and amino acids (Blakely, 1969). These precursors, which are used for the synthesis of DNA, RNA and proteins are required for cell survival.

Figure 5 and 6 show the pathways that require folate coenzymes. In the thymidylate synthesis cycle, the major source of dTMP, dTMP is generated from dUMP and the reduced folate 5,10-methylene-tetrahydrofolate (5,10-methylene-THF) in a reaction catalysed by the enzyme thymidylate synthase (TS). Dihydrofolate (DHF) also generated in this reaction is subsequently converted to THF by dhfr. THF then receives a methyl group from serine to regenerate 5,10-methylen-THF (see Figure 5). This reaction resulting in the formation of glycine is the biosynthetic pathway of this non-essential amino acid.

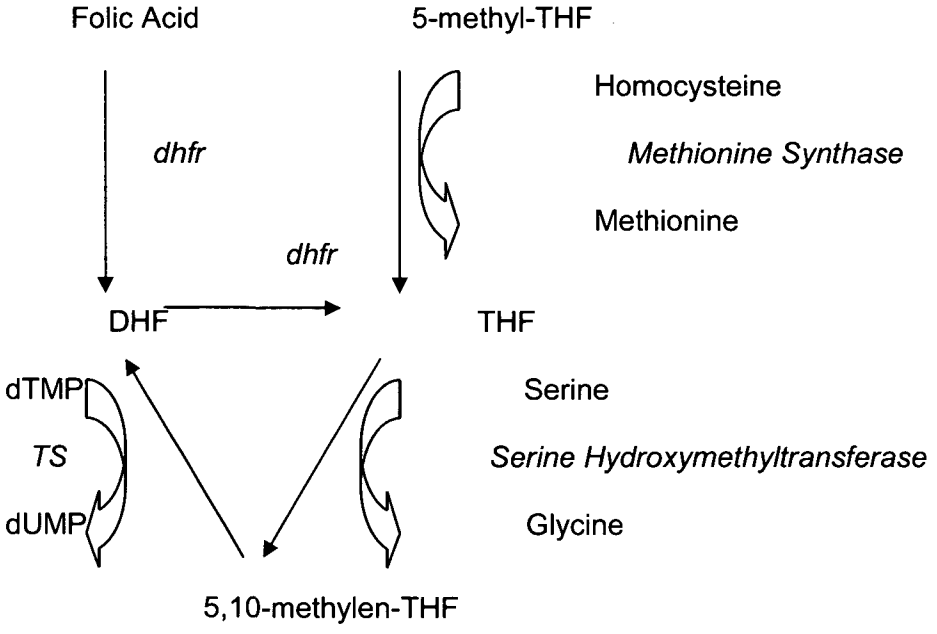

Figure 5: Folate metabolic pathways (Murray, 1997): THF (tetrahydrofolate), DHF (Dihydrofolate), dhfr (dihydrofolate reductase), TS (thymidylate synthase)

Folate coenzymes are also involved in other biosynthetic pathways, such as the synthesis of purine nucleotides (see Figure X). The synthesis of purines from ribose-5-phosphate (R5P) is a multistep pathway that requires various enzymes. Two of them are folate dependent enzymes that catalyse the transfer of one-carbon units from reduced folate substrates to the nucleotide precursors and regenerate THF.

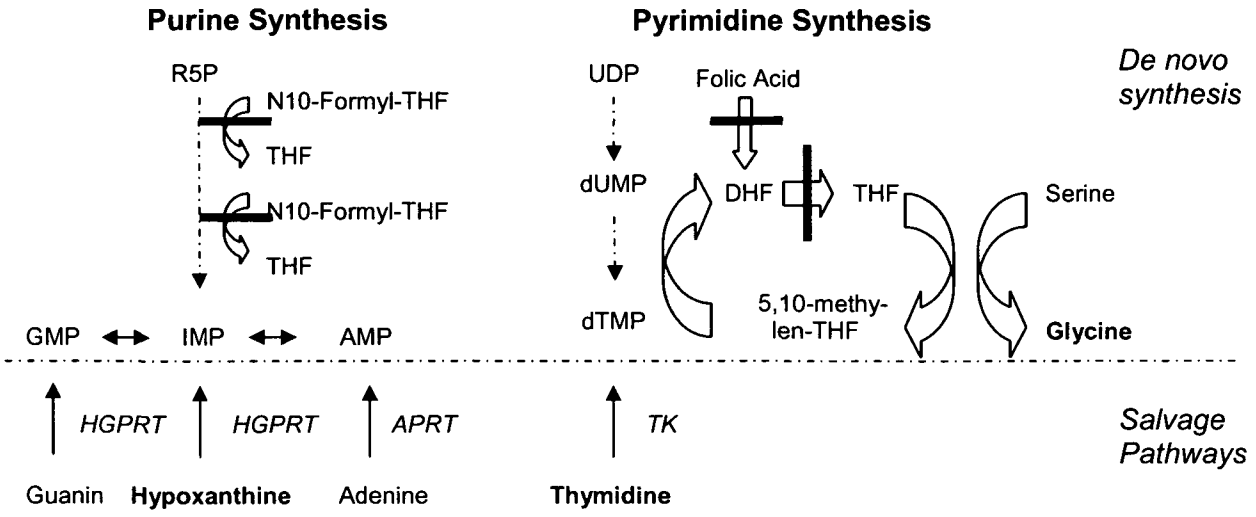

Figure 6: De novo and salvage pathways for biosynthesis of purines and pyrimidines. Dhfr negative cells require hypoxanthine, thymidine and glycine (in blue). Reactions inhibited by folate analogues are shown with red bars.

Due to the metabolic pathways described above, it is clear that dhfr negative cells require the presence of thymidine and hypoxanthine as purine precursors as well as glycine to complement the dhfr deficiency, as the salvage pathway is still functional when a cell lacks de novo synthesis.

Methotrexate (MTX), an analogue of folic acid, was shown to inbibit dhfr (Osborn et al, 1958; Zakrzewski et al, 1960) due to a very high affinity to the enzyme (Werkheiser et al, 1961). By inhibiting dhfr, MTX blocks the reduction of folic acid to DHF and the generation of THF from DHF that is produced during the thymidylate synthesis cycle. That further results in an accumulation of DHF and a depletion of the THF cofactor, which leads to the suppression of folate dependent DNA, RNA and amino acid synthesis and finally results in cell death due to dTMP starvation (Gorlick et al, 1996). Due to its cytotoxicity, MTX has been successfully used to treat various malignancies.

During investigation of selection of mammalian cells for resistance to MTX, the mechanism of dhfr gene amplification in order to overcome the inhibition of MTX was demonstrated (Alt et al, 1978).

The general procedure for the construction of gene-amplified cell lines for the high-level expression of exogenous genes is shown in Figure 7. The first step is the introduction of an amplifiable vector which contains not only the amplifiable and selectable marker gene but also the objective cDNA target gene.

Generally, a cell line that is deficient in or has weak activity of the amplifiable marker enzyme is used as the host cell line. The second step is the gene amplification itself. The transfected cell line ordinarily contains one copy or a low copy number of the transfected gene. In the second step, the transfected marker gene is selectively amplified by the stepwise selection – together with the nearby integrated target gene. Gene amplification is generally achieved by stepwise selection, and high degrees of amplification (10 or more copies) are obtained after the multiple selection. During stepwise selection the concentration of inducers increases and the gene-amplified cell population outgrows the general population.

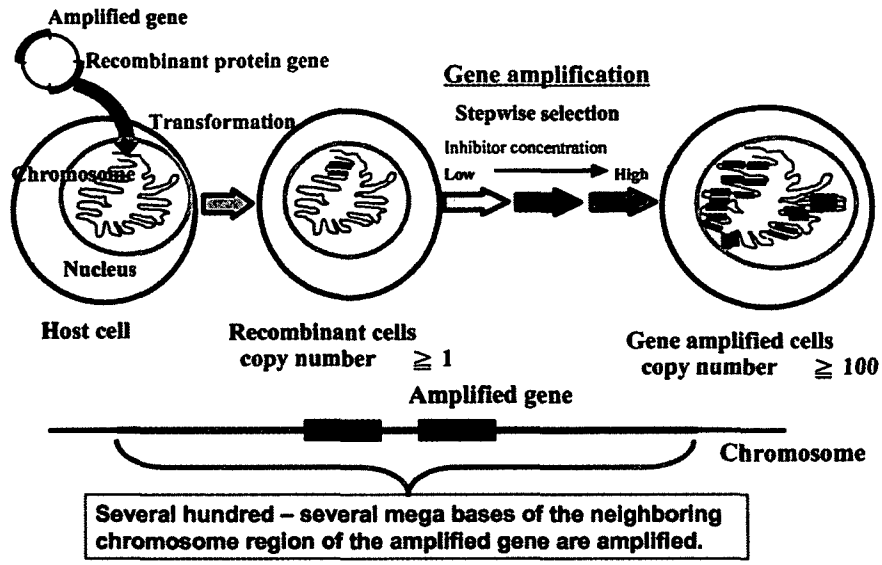

Figure 7: General procedure for the construction of a gene-amplified cell line for high-level expression (Omasa, 2002)

Beside gene amplification, other drug resistance mechanisms are observed during the selection process. For example, single-step high-level resistance to MTX may result in cells with an altered MTX-resistant dhfr enzyme or in cells exhibiting altered MTX transport properties (Kaufman, 1990; Weidle et al, 1988). The genetic background of gene amplification was discovered after Giemsa-Banding, when abnormal chromosome structures were observed: chromosomal regions that failed to exhibit trypsin-Giemsa bands and hence were termed "homologous staining regions" (HSRs) and acentric chromatin bodies referred to as "double minute chromosomes" (DMs). The mechanisms leading to these structures are proposed on one hand in the "breakage-fusion-bridge cycle model"

(leading to DMs) and on the other hand in the "unequal sister chromatide exchange model" resulting in HSRs (Omasa, 2002). Beside these two models, other molecular mechanisms for gene amplification have been proposed.

The GS system owned by Lonza Biologics is predominantly used together with the NS0 cell line which is defective in GS (Bebbington et al, 1992; Barnes et al, 2000). This enzyme is crucial in order to transform glutamate and ammonia to glutamine, which is important in protein, purine and pyrimidine synthesis, ammonia formation, the biosynthesis as well as the degradation of amino acids. Furthermore, the carbon chain of glutamine can serve as a significant energy source for cells.

The incorporation of a GS gene in a plasmid vector containing the gene of a heterologous protein allows selection of cells in glutamine-free media, that have taken up the plasmid during transfection and are stably expressing the GS gene and hence the heterologous protein. The GS coding sequence is usually under the control of a weaker promoter (e.g. SV40, which was proven to be slightly weaker than CMV; Zarrin et al, 1999), however, the heterologous protein coding sequence is often under the control of a powerful CMV promoter (Brown et al, 1992; Keen et al, 1996) ensuring transfectants that survive in glutamine-free media using the weakly-transcribed GS gene and produce high levels of the target protein due to the powerful CMV promoter. Therefore generation of high-level production lines can be frequently achieved even without amplification. Furthermore, due to the transformation of glutamate and ammonia to glutamine, ammonia, an undesirable waste product inhibiting growth, is withdrawn from the culture. A metabolic inhibitor of GS, methionine sulfoximine (MSX) enables greater selection stringency to transfectants and further amplification of the exogenous genes.

As the dhfr system was already established at both industrial partners of the ACBT and due its unrestricted use, the dhfr system was also used in the framework of this thesis. Only few methotrexate amplification steps were applied in order to obtain stable and high producing clones. The serum-dependent recombinant EpoFc clones were selected at a methotrexate concentration of 0.1 μM MTX and selection of the subclones was performed at 0.4 μM MTX. In case of EpoFc cell lines obtained from the protein-free protocol, the transfection pools were cultivated at 0.1 μM MTX and selection of the subclones was done at 0.2 μM MTX.

Vectors

A variety of mammalian-based vectors are used to produce recombinant proteins and monoclonal antibodies. Integration of DNA into mammalian chromosomes is primarily a non-targeted event mediated by non-homologous end-joining into random sites of DNA breakage within the genome (Manivasakam et al, 2001).

An expression cassettes consist of the following components:

Promoters are varying arrangements of short regulatory sequences (e.g. TATA box) for transcription factor and RNA polymerase transcription complex binding located 5′ of the multiple cloning site (MCS), into which the transgene is inserted. The most commonly used promoter is the cytomegalovirus (CMV) major immediate early promoter, which is claimed by the University of Iowa Research Foundation (US Patent 5168062 and 5385839). Their use is permitted for research purposes only and any other use requires a license from the University of Iowa Research Foundation.

The simian virus 40 (SV40) immediate early promoter and the Rous Sarcoma Virus (RSV) long-terminal-repeat (LTR) promoter are also used frequently in expression cassette development. The human Ubiquitin C (UbC) promoter also directs high level recombinant protein expression in a variety of mammalian cell lines. A rather new promoter is the CHO-derived elongation factor-1 promoter (CHEF-1; Running Deer et al, 2004). An example for an inducible promoter (in contrast to the constitutive promoters) is the stress-inducible GADD153 promoter (de Boer et al, 2004).

3′ untranslated regions (UTRs) contain one or more polyadenylation (polyA) sequences (with consensus AATAAA from animal viruses or mammalian genes) and a GT-rich downstream sequence element for transcription termination and polyadenylation of the mRNA 3′ end. Proper termination dramatically affects the stability of an RNA product and facilitates the transport of processed mRNA from the nucleus to the cytoplasm. At minimum, the 3′-UTR requires one or more polyA sequences.

Enhancer elements can further elevate gene expression often independent of their orientation and are often located at a considerable distance from the regulated gene. Viral promoter regions (e.g. CMV and SV40) contain enhancer elements that are frequently included when using these promoters in

expression cassettes. Mammalian genes typically possess multiple intergenic sequences spaced between the coding sequences. They have considerable importance in maintaining the integrity of coding regions, protecting them from mutation, rearrangement or deletion. Furthermore they appear to influence transcription efficiencies and processes involved in pre-mRNA splicing of introns facilitate RNA transport form the nucleus or enhance RNA stability.

Furthermore vectors often consist of **mammalian-selectable markers** (antibiotic, dhfr, GS), **translation control elements**, **5′ UTRs** beside **prokaryotic origins of replication (ORI)** and selectable markers (e.g. ampicillin resistance gene) to make a plasmid an E. coli shuttle vector.

An additional element that has been included in some cassettes is the **internal ribosome entry site (IRES)** typically derived from viral sources. This element enables expression of a single bicistronic mRNA encoding both the gene of interest and a selectable marker. Therefore a single expression cassette can drive expression of both genes.

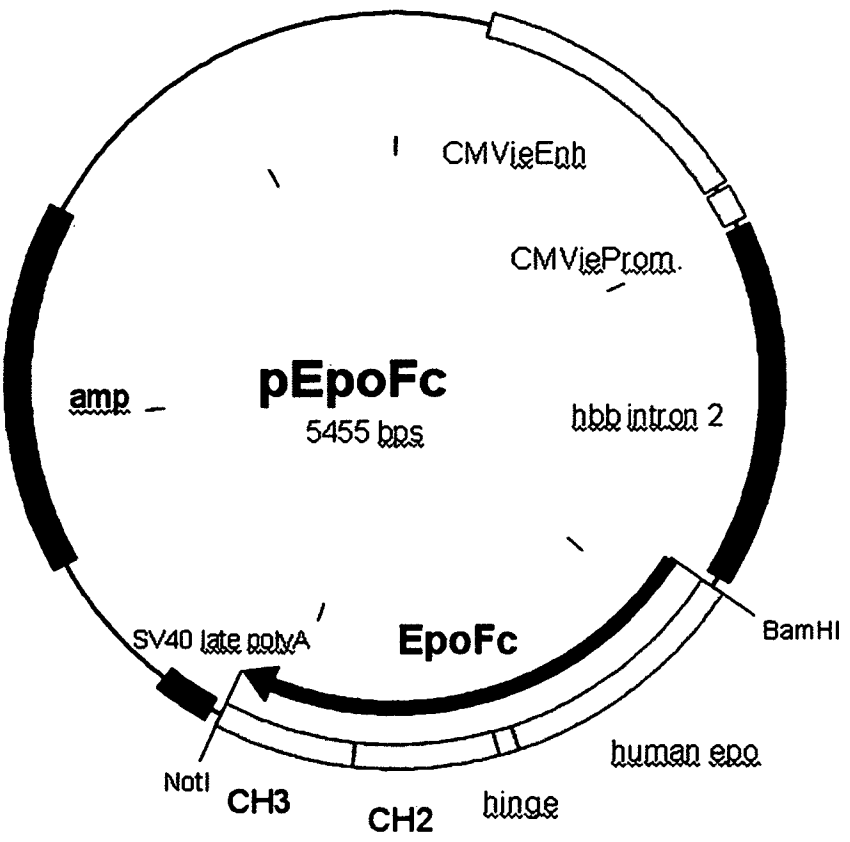

Figure 8: pEpoFc used for expression of the fusion protein

In the case of our expression vector, pEpoFc, the strong constitutive CMV promoter was used (see Fig 8), although we initially started with 3 different promoters: CMV, SV40 and UbC. Due to mutations detected in plasmids with SV40 and UbC promoters leading to a change in the amino acid sequence of EpoFc, we decided to use EpoFc under control of the CMV promoter for our experiments. The polyA sequence was derived from SV40. The selection marker dhfr was expressed from a second plasmid after co-transfection of the two vectors.

Novell Strategies for Enhancing Productivity

Major advances have been made over the past decades in the use of mammalian cells for recombinant protein production – nevertheless a big potential for optimising productivity and stability still remains in the regulatory events in the production pathway which can be divided into three phases (Barnes et al, 2006) – the chromosomal environment, mRNA stability and processing as well as translation and secretion events.

Chromosomal Environment

Much of the variability encountered in productivity levels and stability is associated with chromosomal transgene integration sites (Kayser et al, 2006). Integration into tightly packed and heavily methylated heterochromatin is thought to lead to lower levels of productivity and greater risk of gene silencing - in contrast to integration into the more open and active euchromatin containing gene-rich regions. This phenomenon known as the "position effect" is a major hurdle in recombinant protein production and specifically, in cell-line generation (Wurm, 2004). In addition, integrated transgenes tend to be silenced over time due to epigenetic modifications of DNA (methylation) and histones (acetylation, methylation, phosphorylation and ubiquitination). In the recent decade, researchers have taken different approaches to overcome these problems: One approach is to use targeted integration into previously identified transcriptional "hot spots", another is the use of chromatin structure elements in order to create an environment around transfected genes that will favour active transcription. For this purpose, either the surrounding environment is prevented physically from influencing transcription or the overall epigenetic environment of the surrounding chromosomal DNA is altered. The following **chromatin structure elements** are among the well-characterised *cis*-acting elements that have been incorporated into vectors:

- **Insulator and barrier elements** prevent cross-regulation of adjacent genes or gene clusters by restricting the activity of enhancers and silencers (Kellum et al, 1991; West et al, 2002).
- **Matrix/Scaffold attachment regions (S/MARs**; e.g. chicken lysozyme 5′ MAR elements; Girod et al, 2005) are AT-rich DNA elements that attach chromatin fiber to the nuclear matrix

or scaffold. Researchers proposed that S/MARs regulate gene expression by organizing chromatin into separate loops or act as insulators or chromatin domain openers (Schubeler et al, 1996; Heng et al 2004).

- Locus control regions (**LCRs**) enhance expression (Li et al, 1999)
- Ubiquitous chromatin opening elements (**UCOEs**) derived from the promoters of housekeeping genes exhibit nontissue–specific preventive activity against transcriptional silencing (Benton et al, 2002)
- **Antirepressor or STAR (stabilising and antirepressor) elements** are used to flank transgenes in expression vectors and affect the spread of methylation and histone deacetylation patterns from the surrounding genome into the recombinant DNA (Kwaks et al, 2003).

In order to investigate the genetic environment adjacent to a short stretch of known sequences exogenous elements, the technique of targeted gene walking polymerase chain reaction (PCR) has been developed. By the use of a single, targeted sequence specific PCR primer with a second, non specific "walking" primer the unknown flanking regions can be identified (Parker et al, 1991). Further optimisation of this method has led to isolation of larger upstream fragments (Rishi et al, 2004).

Methods to alter the general epigenetic environment include the acetylation of histones (Kwaks et al, 2005) which is generally associated with enhanced transcription due to the balance of histone acetyltransferase (HAT) and histone deacetylase (HDAC) activies and the prevention of deacetylation using HDAC inhibitors such as sodium butyrate (Davie, 2003). Due to its potentially cytotoxic effects on mammalian cells, sodium butyrate has been used as part of a combinatorial treatment together with overexpression of antiapoptotic factors such as Bcl-2 (Kim et al, 2003) or down regulation of pro-apoptotic components like caspase-3 using antisense RNA technology (Kim et al, 2002).

mRNA Stability and Processing, Translation and Secretion

In contrast to the chromosomal environment, mechanisms of controlling mRNA expression as well as translation and secretion are still poorly defined (Barnes et al, 2006). Now total gene synthesis is often used, that allows the use of codon-optimisation and the elimination of RNA secondary structures. Recently, a specific RNA processing event has been shown to have unique consequences for host cell productivity. This relates to alternate splicing of the transcript for the transcription factor XBP-1 (X-box binding protein) that can either lead to secretory expansion or to activation of apoptosis and decreased cell biomass (Yoshida et al, 2006). Another study (Tigges et al, 2006) demonstrates that expansion of the endoplasmic reticulum and the Golgi of transgenic Chinese hamster ovary (CHO-K1)-derived cell lines with a resulting increase in overall production capacity could be achieved by using vectors containing XBP-1 in its potent, spliced form XBP1-(s). XBP1-(s) has emerged as a master regulator coordinating sustained stress recovery across the entire endomembrane/endocytic systems.

Recombinant proteins that are also naturally secreted from the cell are more straightforward to produce as it is also secreted by the host and can be purified from the medium. Problems occur if the recombinant protein is a naturally non-secreted protein. Efforts have been undertaken in order to either enhance the secretion of regularly secreted protein or to induce secretion of a normally non-secreted protein after exploitation of genetic signals that determine the posttranslational fate of the nascent protein and the protein secretion machinery of host cells.

The protein synthetic apparatus is compartmentalised into at least three polysome populations - free, cytoskeletal-bound and membrane-bound polysomes. Different signals direct the mRNAs to their site for translation. In the case of membrane-bound and secreted proteins it has been well established that the signal peptide is required to target the ribosome-mRNA complex to the endoplasmic reticulum (ER) whilst it has been shown that for mRNAs localised in the cytoplasm or associated with the cytoskeleton there are signals in the 3'UTR region (Russo et al, 2006).

Signal peptides, comprising the N-terminal 13–36 amino acids of secretory proteins, are necessary for the translocation across the first membrane on the secretory pathway and thus universally control the

entry of all proteins to the secretory pathway in eukaryotes and prokaryotes. Although signal peptides are not highly conserved, they have a common positively charged n-region, a hydrophobic h-region and a neutral, polar c-region. In contrast, leader sequences are polynucleotide regions located between the promoter and the coding region and are involved in the regulation of gene expression.

Part of the leader sequence may be translated into a short leader peptide but, in contrast to signal peptides, leader peptides are at no time part of the structural proteins. Leader sequences comprise a short open reading frame coding for a leader peptide and a downstream adjacent region with the propensity of forming mutually exclusive secondary structures (stem-loops) by base-pairing of complementary sequences. The formation of one or the other possible stem-loops depends on stalling of the ribosome during translation of the leader peptide, either because of lack of the necessary tRNA or because of binding of a specific metabolite to the ribosome/mRNA complex. In turn, the formation of the alternative mRNA conformation affects either the continuation of transcription (transcriptional attenuation) or the initiation of translation of the protein coding region (translational attenuation). Thus, leader sequences may regulate gene expression at the level of transcription or translation.

However, although signal peptides and leader sequences clearly represent different entities and have different functions they are often used synonymously to depict signal peptides.

As soon as the signal peptide of a nascent precursor protein (pre-protein) has directed the ribosome to the rough ER membrane, the signal sequence is recognised by complexes in the ER membrane known as signal recognition particles (SRP's). Upon binding to a SRP the translation of the polypeptide is halted until the ribosome-SRP-signal peptide complex has bound to the docking protein (SRP-receptor) at the ER membrane.

The signal peptide is then inserted into the ER membrane via a signal peptide binding protein and the nascent polypeptide then crosses the ER membrane through a transmembrane channel. During the translocation across the ER membrane in eukaryotes, the signal peptide is normally cleaved off the pre-protein by a signal peptidase residing in the ER or cytoplasmic membrane. After passing through the ER and the Golgi apparatus proteins are being packaged into membrane bound vesicles that allow secretion.

Many efforts have been undertaken in order to determine signal peptides that enhance protein secretion. UTRtech™ offers the use of a signal peptide derived from *Gaussia princeps* luciferase. In contrast to the use of the albumin signal peptide, secretion of a normally unsecreted protein could thus be raised from 3 % up to 100 % (DE69836336D). Further potential might be found in altering the 3'UTR as well as the 5'UTR that flank the signal peptide as well as the protein coding region. A recent study has identified an eleven nucleotide section in the 3'UTR that is required for localisation of rat metallothionein-1 mRNA (MT1) in the perinuclear cytoplasm (Nury et al, 2005). Such mRNA targeting is believed to provide a mechanism for local synthesis of proteins close to where they function (Hesketh, 1994).

Bamford et al (1998) demonstrated that IL-15 expression is transcriptionally impeded not only by the 5'UTR, but also by the coding sequence of its signal peptide and its mature protein carboxyl terminus.

Beside the pathways involved in increasing productivity such as transcription, translation, posttranslational modification, and secretion, genetic enhancements to increase growth (and productivity indirectly) include protooncogene and growth-factor–related pathways, pathways to alter cell cycle control, and components to alter the apoptotic state of a culture.

Proper folding and posttranslational modification of a recombinant protein in the endoplasmic reticulum (ER) is an important factor in achieving the desired function of a recombinant protein. Increasing the efficiency of folding reactions has been attempted in several studies with molecular chaperones and PDI (protein disulfide isomerase, the enzyme responsible for disulfide bond formation in MAbs) have achieved limited success in improving protein folding.

Finally, a finished protein must be secreted appropriately to be used in most commercial programs. All these functions are regulated by the unfolded protein response (UPR) pathway, which senses an abundance of translated protein in the ER and increases secretory machinery to accommodate the load while reducing levels of translation (Schroeder et al, 2005).

In the end, highly productive clones are achieved through a synergy of cell line engineering methods, clone selection, and media optimization efforts.

Targeted Integration

Targeted integration methods should be considered as a future alternative to random integration approaches. Targeted integration into transcriptionally active regions of chromosomes could potentially overcome positional effects and regional instability issues.

During our studies, we investigated the integrations site of the exogenous targets in a variety of recombinant CHO cell lines by fluorescence in situ hybridisation (FISH) in order to gain information of insertion loci that enable high and stable protein expression.

Several novel technologies integrate into specific regions. The most common is the use of recombinases such as the Cre-Lox and FLP systems (Wirth et al, 2004). These sites are placed in regions of the chromosome that can be retargeted with different transgenes (Bode, 2003) once a transcriptionally active hotspot has been located using site-specific recombinases. Other recently targeted technologies include the use of zinc finger nucleases (Porteus et al, 2005; Porteus, 2006) to insert genetic material into a host's chromosome. Artificial chromosomes provide for a novel approach to integrate transgenes such as the satellite DNA-based artificial chromosome (ACE; Vanderbyl et al, 2005).

Screening

At current, screening for the future production clone is predominantly based on specific productivity, growth, stability and activity of the product of the early clones. In order to determine the best producer, several methods have been developed in order to accelerate the screening process. Fluorescence activated cell sorting (FACS) allows rapid isolation of clones with large amounts of secreted product passing the cell membrane. Nevertheless, FACS does not necessarily yield high-producing cell lines due to a lack of correlation between cell membrane and secretion. A rather novel method is ClonePix^{FL} technology (Genetix), which uses in situ identification and measurement of cell-secreted proteins to identify and isolate appropriate cell colonies by trapping the product with fluorescently labelled antibodies.

Especially the third parameter, stability of the selected clone, is very important in order to avoid the risk of instabilities in the large-scale production process. Determination of stability is very time-consuming as productivity of the cell line has to be followed over several weeks and does not always guarantee stable expression when the clone is subjected to new culture conditions, e.g. the shear stress in the bioreactor. Several studies have investigated integrations sites that lead to stable expression (e.g. Yoshikawa (2000a and b) demonstrated, that integration into telomeric regions favours stability and productivity) by FISH analysis, but yet no other method for selecting stable clones is available.

In the framework of our studies, we tried to focus on the genetic level and analysed gene and mRNA copy numbers by quantitative PCR (qPCR) in order to determine parameters that allow prediction of clone behaviour. Furthermore we investigated insertion sites of a broad range of stably producing recombinant cell lines in order to gain additional information on parameters that influence stability.

SELECTED UNPUBLISHED WORK

Comparison of Quantitative PCR methods

Gene copy numbers were determined by Southern blot and by quantitative PCR after comparison of two different methods for quantification – the Sybr-Green system, in which the intercalating dye binds to every double stranded DNA molecule, and the Taqman technique, where a complementary probe specifically binds to the gene of interest.

Material and Methods

Five primary EpoFc transfectants (2C10, 2G4, 2G6, 8C6 and 10D9) as well as one sub-clone of 2C10, namely 2C10/13F5 (see Lattenmayer et al, 2006c for details) were analysed for gene copy numbers by quantitative PCR and Southern blot.

For preparation of genomic DNA $2.5 \cdot 10^6$ cells were washed in PBS (without Ca^{2+} and Mg^{2+}, pH 7.2), nucleic acid isolation was performed by using the QIAamp® DNA Blood Mini Kit (Qiagen) according to the manufacturer's instructions. Concentration of DNA was determined by measuring the absorption at 260 nm with a BioPhotometer (Eppendorf). Genomic DNA samples were adjusted to 20 ng/µl and stored at +4 °C.

For Southern blot analysis a DIG-labelled Fc specific amplicon (581 bp) was generated by PCR using PCR DIG Labelling Mix (Roche), Isis™ DNA Polymerase (Qbiogene) and sense primer 5′-GGGACCGTCAGTCTTCCTCT-3′ and antisense primer 5′-CATGCATCACGGAGCATGAG-3′ (Genxpress). The DIG labelled probe was purified by QIAquick PCR Purification Kit (Qiagen) and quantified spectrophotometrically. 1.5 µg BamHI digested genomic DNA of each clone as well as 0.01, 0.1 and 1 ng of plasmid DNA standards (pEpoFc; 1 ng = $1.67 \cdot 10^8$ copies) were separated on a 1% agarose gel and transferred to the positively charged nylon membrane according to the Roche Manual. After prehybridisation (7% SDS, 50% Formamide, 5×SSC, 2% Blocking Reagent, 50 mM Sodium-phosphate pH 7.0, 0.1% N-Lauroylsarcosine), the membrane was incubated in hybridisation buffer containing 10 ng/ml of the denatured probe for at least 12 hours at 42 °C. Washing and

detection was performed as described by Roche using Anti-Digoxigenin-AP Fab fragments (Roche) and CPD-Star® (Tropix). Visualisation was done on the LumiImager™ (Roche).

The Sybr-Green Method and the Taqman quantitative PCR were compared in the Rotor-Gene 2000 (Corbett Research). The plasmid standard pEpoFc (5455 bp, stock solutions of $1 \cdot 10^8$ copies/µl stored at -20 °C) was used in a range from $3 \cdot 10^6$ to $3 \cdot 10^3$ copies in a 1:10 dilution series analysed in duplicates. PCR grade water (Roche) was used for all dilutions and reactions. 6 ng of genomic DNA and 0.6 ng of cDNA were analysed on the FAM/SYBR Channel CH1 (495/620 nm) and results were evaluated with the Rotor-Gene Software V 5.0 at a threshold of 0.06.

Sybr-Green Method: 2.5 pmol of each primer (sense 5′-CGTGGAGTGGGAGAGCAATG-3′, antisense 5′-CATGCATCACGGAGCATGAG-3′) designed by the Primer3 Software (X) and iQ Sybr-Green Supermix (Biorad) were used in a total reaction volume of 10 µl. The resulting amplicon (155 bp in length) was generated by the following cycling parameters: 95°C for 5 min, then 45 cycles of 95°C for 15 sec, 58°C for 5 sec, 72°C for 15 sec, with a final recording step at 77°C for 3 sec.

Taqman Approach: We used iQ Supermix (Biorad), 18 pmol of each primer (sense 5′-TCCTTCTTCC TCTACAGCAAGC-3′, antisense 5′-ATCACGGAGCATGAGAAGACG-3′) and 3 pmol of the fluorogenic probe (5′-TGCTGCCACCTGCTCTTGTCCACG-3′) labelled with 6-carboxyfluorescein (FAM) at the 5′ end and the fluorescent quencher dye 6-carboxytetramethylrhodamin (TAMRA) designed by Primer Express Software in a total reaction volume of 10 µl. A 77 bp amplicon was obtained after PCR by using the following cycling parameters: denaturation (95°C, 5 min) and cycling (45 cycles) consisting of 95°C for 15 seconds and 60°C for 1 minute (recording at the end of the second step).

Results and Discussion

Despite poor information in the literature on quantification of Southern blot, we determined the GCN by a standard curve (1, 0.1 and 0.01 ng per lane representing 170, 17 and $1.7 \cdot 10^6$ copies) based on the Boehringer Luminescence Units [BLU] detected by the LumiImager using the LumiAnalyst 3.0 Software (Filtered Profile, 10 %).

The calculation of the target gene per cell was based on the size of the hamster genome ($3 \cdot 10^9$ bp) resulting in 3.3 pg DNA per cell, indicating that 1.6 µg of genomic DNA contained $4.9 \cdot 10^5$ copies of the genome. After calculating the amount of target via the equation of the standard curve, the gene copy number was determined by dividing this result by the amount of genomes loaded on the blot. Results of four analyses are averaged in Table 4. In case of results < 1 copy/cell, a gene copy number of at least one was estimated due to the clear signal on the blot and the general low sensitivity of blot methods.

Clone	GCN Blot 1 [copies/cell]	GCN Blot 2 [copies/cell]	GCN Blot 3 [copies/cell]	GCN Blot 4 [copies/cell]	Average GCN [copies/cell]	Standard Deviation [%]
2C10	1	4	11	1	4	159
2G4	2	1	1	1	1	79
2G6	1	2	6	1	2	124
8C6	1	1	1	1	1	0
10D9	137	84	113	134	117	30
2C10/13F5	5	20	38	10	18	82

Table 4: Calculation of the Gene Copy Number based on four analyses: Specific gene copy number (GCN) was calculated based on the amounts of a hamster genomes loaded onto the blot.

Several studies claim that blot methods are less sensitive (Malinen, 2003; Bustin, 2000; Dean, 2002), especially when investigating low copy numbers, which can also be observed for clones 2C10, 2G4, 2G6 and 8C6 (see Table 8). Therefore the use of quantitative PCR in order to determine gene copy numbers of clones is recommended, especially when investigation of clones without extensive MTX amplification is required.

The gene copy number of EpoFc was quantified with Sybr-Green and Taqman quantitative PCR by analysing 6 ng genomic DNA of each clone (representing 1818 molecules of the genome). Genomic DNA from two passages of each clone was analysed three times and the GCN was averaged from six results. Both analyses showed the same order of gene copy numbers of the clones (correlation coefficient 99.8 % between the results of the two methods, see Figure 9), although results of the Taqman method were 2.5 times higher than the amounts obtained with the Sybr-green method (see Table 5).

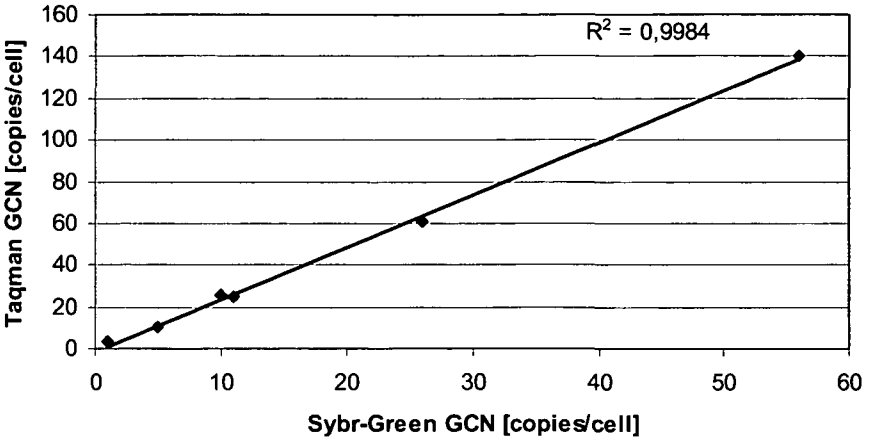

Figure 9: Correlation of gene copy numbers determined by Taqman and Sybr-Green quantitative PCR.

In case of both methods, standard deviations were in the range of 20-40 % except for the clone with the lowest gene copy number, 8C6, were 140 % of standard deviation were observed with the Sybr-green method, and in case of clone 10D9, showing the highest gene copy number, where only 5 % standard deviation were reached with the Taqman method.

Clone	Sybr-Green		Taqman	
	GCN per cell [copies/cell]	Standard Deviation [%]	GCN per cell [copies/cell]	Standard Deviation [%]
2C10	10	30	26	19
2G4	5	37	10	30
2G6	11	18	25	12
8C6	1	142	3	37
10D9	56	39	140	5
2C10/13F5	26	42	61	28

Table 5: Calculation of the Gene Copy Number: Samples were analysed three times, results from two passages were averaged.

Figure 10 shows a summary of the GCNs obtained after analysis by Southern blot and quantitative PCR. Standard Deviations in Southern blot were the same as observed in Sybr-Green quantitative PCR for clone 8C6 bearing the lowest gene copy number and furthermore up to four times higher than in Taqman quantitative PCR, where values of 5-40% were observed.

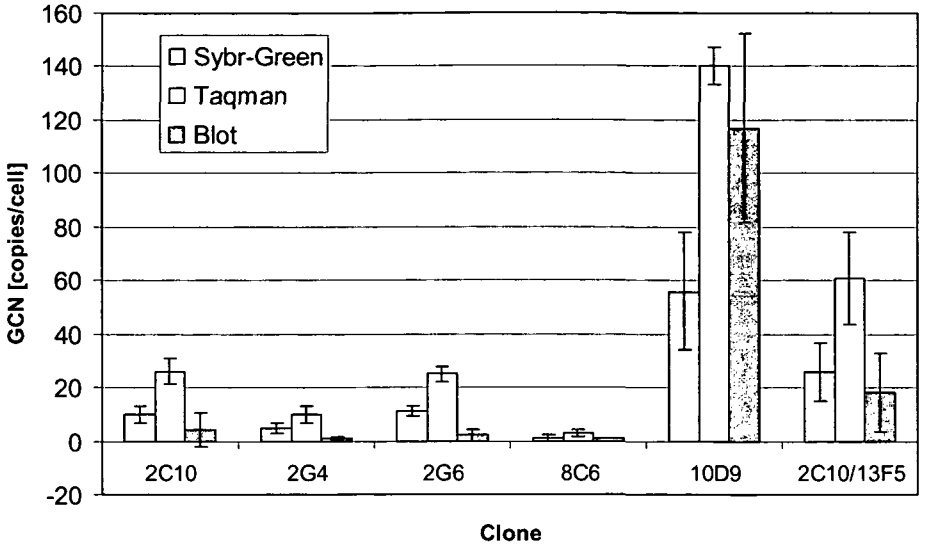

Figure 10: Gene copy numbers determined by three methods. Highest results as well as lowest standard deviations were obtained with the Taqman quantitative PCR.

The factor of 2.5, which we observed after comparison of the two qPCR methods, was also reported when analysing same samples with different lots of reaction mixes (Bustin, 2002) and in addition, Bubner (2004) claims that two-fold differences in GCN are the detection limit in quantitative PCR. The problems of quantitative PCR arise predominantly from the back transformation of the linear model to real data, since quantitative PCR follows exponential growth kinetics.

The Taqman detection method uses a probe that hybridises to the correct amplicon and is therefore highly specific, while lower variation coefficients are reported in the Sybr-Green method (Wong, 2005; Pfaffl, 2004). We determined efficiencies and correlation coefficient of the standard curves and found an average efficiency of 0.73 and a correlation coefficient of 0.97 for the Sybr-green standard curves and a higher average efficiency of 1.00 and a correlation coefficient of 0.99 for the Taqman standard curves. Furthermore, standard deviations of the results were far better when analysing GCN by the Taqman method, especially when looking at clone 8C6 having the lowest GCN. Based on these data and the need for analysis of very low copy numbers with high sensitivity, we decided to continue our experiments with the Taqman PCR.

CONCLUSION

Beside the development of a protein-free protocol for cell line generation (Lattenmayer et al, 2006a) extensive characterisations of clone performance (productivity, stability) and genetic parameters (GCN, mRNA levels; Lattenmayer et al, 2006c) have been performed. Furthermore, the insertion loci of the exogenous genes were determined in our EpoFc cell lines as well as in several other antibody and protein producing recombinant CHO cell lines by FISH analyses (Lattenmayer et al, 2006c). Figure 11 gives an overview of the recombinant EpoFc clones that were generated and investigated in the framework of this thesis:

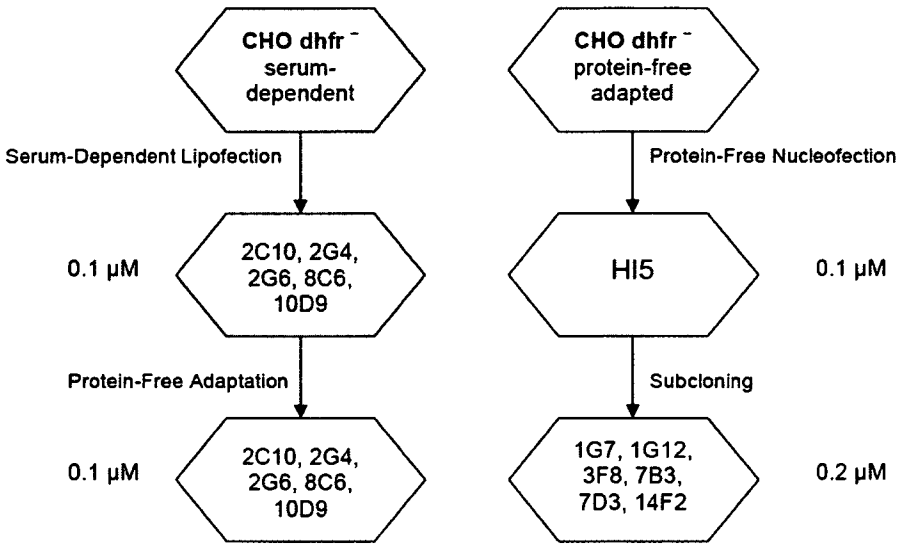

Figure 11: Overview of the recombinant EpoFc clones. Serum-dependent cultivation is shown in grey whereas protein-free clones are shown in white colour.

Table 6 summarises the specific productivities as well as the genetic parameters of the different clone families. Although higher specific productivities can be observed for the subclones of the serum-dependent transfectants, comparable values are evident for the clones obtained after subcloning of the transfected pool and the protein-free adapted clones that were seeded into 96-well plates already immediately after transfection.

The loss of specific productivity during protein-free adaptation (that is not only observed for our clones) further justifies the use of a protein-free transfection protocol as productivity and stability parameters for determinating the suitability of a clone to become a production clone might not be valid after protein free adaptation.

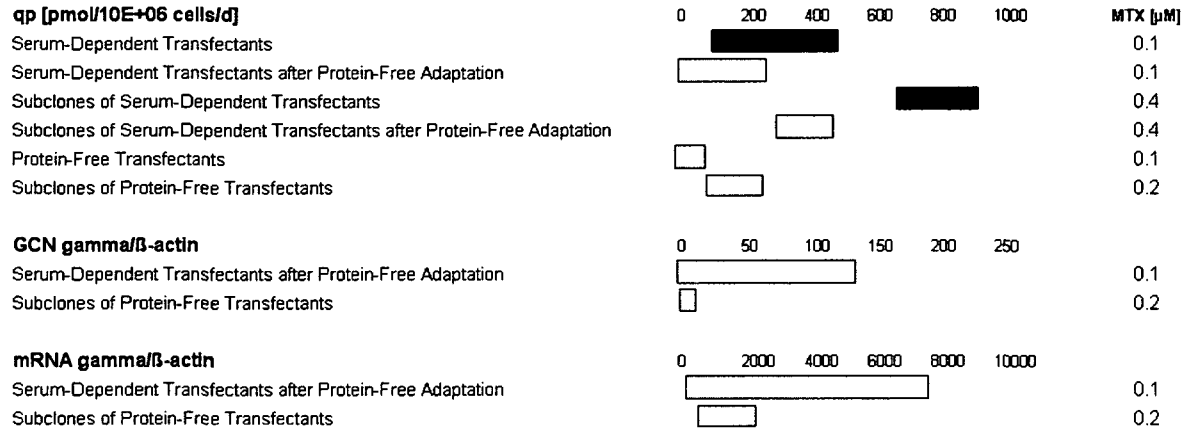

Table 6: Overview of specific productivity (1 pmol EpoFc = 0.112 µg based on an estimated molecular weight of 112131.2 Da) and genetic parameters of the different EpoFc clone families.

Lots of results have been obtained from the genetic investigations. To summarise, a good correlation of mRNA with specific productivities was obtained although a high gene copy number is not necessarily reflected in a high specific productivity. Comparing the serum-dependent clones after protein-free adaptation to the subclones of the protein-free transfectants, higher GCNs as well as mRNA levels are evident for the protein-free adapted clones, although specific productivities are comparable. Whether this is due to a less efficient translation efficiency already immediately after transfection or became evident as a consequence of protein-free adaptation still remains uncertain. In order to enable further discussion of the suitability of genetic parameters as further selection tools for early transfectants, far more cell lines producing different products have to be analysed.

The investigations of the insertion loci of a broad range of recombinant CHO cell lines resulted in different integration sites of the exogenous gene with a slight preference of larger chromosomes as targets for integration (Lattenmayer et al, 2006c). Due to these observations, several integration sites enabling stable and high productivities exist – the next step therefore is the characterisation of the

genetic environment of these loci which might provide useful knowledge for either targeting these sites as well as integrate potential control elements already in the transfer vector.

Further studies of our recombinant EpoFc cell lines have been performed in the framework of the ACBT in order to improve the efficiency of biopharmaceutical process development. Schriebl et al (2006a) analysed the fusion protein purified from the culture supernatant and quantified N-acetylneuraminic acid, the quantitative isoform and the entire glycan pattern. No significant differences in the core glycan structures compared to rhEpo and human antibody N-glycans were found. Furthermore, the development of an alternative isoform prestaining method with CyDye fluors is described for EpoFc (Schriebl et al, 2006b). This method also enables quantitative isoform pattern analysis directly from a serum-free culture supernatant.

In a systematic approach Trummer et al (2006a) varied the dissolved oxygen tension, pH, and temperature of EpoFc cultures in controlled bioreactors and investigated the impact on growth, productivity, metabolism, product quality and cell cycle distribution and found the reduction of cultivation temperature and the reduction of (external) pH to exert the most significant effects on process performance by mainly reducing cell growth and metabolism. With respect to the cell line used they identified a set of parameters capable of affecting cell proliferation in favour of an increased specific productivity and total product yield. Furthermore, Trummer et al (2006b) investigated these process parameters in a series of biphasic batch cultivation experiments of EpoFc cell lines. In most of these processes the integral of viable cells and the specific productivity were increased leading to a significant improvement of both final product concentration and volumetric productivity. In addition, combined parameter shifts (pH 6.90/ 30 °C and pH 6.90/ 33 °C) exerted a synergistic effect on product quality. The loss of product sialylation which occurred at reduced temperatures was prevented by simultaneously reducing the external pH.

The studies of ACBT II focus on the development of sorting protocol for cell lines with a more efficient energy metabolism using our EpoFc cell lines as model systems; furthermore the work of Trummer et al (2005) on transcriptome analyses of the recombinant EpoFc cell lines will be continued in order to identify novel, cold-shock inducible promoter sequences.

ABBREVIATIONS

ACBT	Austrian Center of Biopharmaceutical Technology
ACE	artificial chromosome
APRT	adenine phosphoribosyl transferase
BHK	baby hamster kidney cell line
BSA	bovine serum albumin
BUdR	5-bromodeoxyuridine
CDM	chemically defined media
cDNA	complementary DNA
CHO	Chinese Hamster Ovary cell line
CMV	cytomegalovirus
COS	monkey kidney cell line
DEAE	dimethylaminoethyldextran
DHF	dihydrofolate
dhfr	dihydrofolate reductase
DM	double minute chromosome
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethylsulfoxide
DNA	desoxyribonucleic acid
DOPE	L-dioleoyl phosphatidylethanolamine
dUrd	deoxyuridine
EGF	epidermal growth factor
Epo	erythropoietin
EpoFc	erythropoietin-huIgG1Fc fusion protein
ER	endoplasmic reticulum
ER	endoplasmic reticulum
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
FDA	food and drug administration (US)
FGF	fibroblast growth factor
FISH	fluorescence in-situ hybridisation
GS	glutamine synthetase
HAT	histone acetyltransferase
HAT-Medium	medium containing hypoxanthine, aminopterin and thymidine
HDAC	histone deacetylase

HEK-293	human embryo kidney cell line
HGPRT	hypoxanthine guanine phosphoribosyltransferase
HIV	human immunodeficiency virus
HSA	human serum albumin
HSR	homologous staining region
IAM	Institute of Applied Microbiology
IRES	internal ribosome entry site
LCR	locus control region
LTR	long-terminal-repeat
MAR	matrix attachment region
MCB	master cell bank
MCS	multiple cloning site
mRNA	messenger RNA
MSX	methionine sulfoximine
MTX	methotrexate
NS0	non-secreting myeloma cell line from mouse
OPD	orthophenylenediamine
ORI	origin of replication
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PDI	protein disulfide isomerase
PEG	polyethylenglycol
PFM	protein-free media
polyA	polyadenylation
qPCR	quantitative PCR
RNA	ribonucleic acid
RSV	rous sarcoma virus
R5P	ribose-5-phosphate
SAR	scaffold attachment region
SFM	serum-free media
Sp2/0	mouse myeloma cell line
SRPs	signal recognition particles
SV40	simian virus 40
THF	tetrahydrofolate
TK	thymidine kinase
TS	thymidylate synthase

TSE	transmissible spongiform encephalopathies
UbC	ubiquitin C
UCOEs	ubiquitous chromatin opening elements
UPR	unfolded protein response
UTR	untranslated region

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ARTICLE

BIOTECHNOLOGY AND BIOENGINEERING

Protein-Free Transfection of CHO Host Cells With an IgG-Fusion Protein: Selection and Characterization of Stable High Producers and Comparison to Conventionally Transfected Clones

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ABSTRACT: In order to improve the current techniques of cell cultivation in the absence of serum, we have developed a protein-free transfection protocol for CHO cells, based on the Nucleofector™ technology. After starting with a heterogeneous pool of primary transfectants which express the fusion protein EpoFc, we isolated single clones and compared them with parallel clones generated by lipofection in serum-dependent cultivation. Our intensive characterization program was based on determination of specific productivity (q_p) and analysis of genetic parameters. In two nucleofection experiments, transfection with 5 μg of DNA resulted in best productivities of the primary cell pools. After subcloning, the q_p could be raised up to 27 pg·cells^{-1}·day^{-1}. While the serum-dependent transfectants exhibited specific productivities up to 57 pg·cells^{-1}·day^{-1} in serum-dependent cultivation, a significant decrease that resulted in the range of q_p of the protein-free transfectants was observed after switching to protein-free conditions. Investigation of genetic parameters revealed higher mRNA levels and gene copy numbers (GCN) for the protein-free adapted serum-dependent transfectants. Therefore, we assume that problems during protein-free adaptation (PFA) lead to a less efficient translation machinery after serum deprivation. We describe the generation of stable-producing recombinant CHO clones by protein-free transfection of a protein-free adapted host cell line, which reduces the risk of adverse clonal changes after PFA. The main advantage of this approach is the earlier predictability of clone behavior, which makes the generation of production clones by protein-free transfection, a viable and highly efficient strategy for recombinant cell line development.

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KEYWORDS: protein-free transfection; nucleofection; EpoFc fusion protein; characterization; CHO; serum-free

Introduction

Selection and screening of recombinant production cell lines for generation of biopharmaceuticals mainly rely on serum-based cultivation, and the switch to serum-free conditions is made before the scale up. The drawbacks of serum use lie in the areas of safety issues, costs, disadvantages in the purification process of the recombinant protein as well as considerations regarding animal welfare. Furthermore, serum is undefined with a high batch-to-batch variability (Morris and Warburton, 1994) and the process of protein-free adaptation (PFA) involves the risk of adverse clonal changes (Ozturk and Palsson, 1990). Therefore, regulatory authorities like EMEA and FDA have encouraged biotechnological manufacturers to reduce or totally eliminate the use of substances of animal origin in their production processes (Castle and Robertson, 1999).

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First efforts to get rid of serum resulted in the development of serum-free media, which still contain animal-derived or recombinant components, including albumin (BSA, HSA), fetuin, insulin, various hormones, growth factors, and other proteins. However, the question of their origin leads to further regulatory demands and the presence of these proteins renders purification of recombinant products difficult, time consuming, and expensive.

To overcome these limitations, protein-free media have been constructed. Adaptation of recombinant CHO cell lines, but also of the host cell line K1 (Puck et al., 1958) or dhfr$^-$ (Urlaub and Chasin, 1980), to serum-free cultivation conditions is easy and only few cell lines are reported where PFA was not successful (Schroder et al., 2004). In contrast, transfection of serum-free host cells was done only transiently (Derouazi et al., 2004; Rosser et al., 2005) and even fewer studies were performed under protein-free conditions (Schifferli et al., 1999). In case of therapeutically applied proteins, material from transient transfection can be used for medical studies and early clinical trials, but the production process and the master cell line must be defined for further studies. Kuchenbecker et al. (2005) showed successful subcloning of a recombinant CHO cell pool generated by nucleofection under serum-free (but still not protein-free) conditions, after selection by hypoxanthine/thymidine deprivation.

Based on these considerations, the need for the establishment of stable transfection methods under protein-free conditions is clearly evident. First efforts have been made with the addition of low amounts of serum during the transfection event. Serum is reported to have a protective effect against apoptosis (Zanghi et al., 1999), mainly due to growth factors, and therefore supports survival of the positive transfectants. As the decrease of viability was the major problem we observed for our serum-independent transfected cells, the transfection conditions as well as the handling of the transfected pools had to be optimized in order to obtain stably producing clones.

In contrast to other non-viral transfection methods that rely on cell division for the transfer of DNA into the nucleus, nucleofection allows the transport of the DNA directly into the nucleus due to optimized electrical pulses and buffer systems (Siebenkotten, US Patent US2004014220). In this study, we demonstrate that nucleofection under protein-free conditions and selection with methotrexate (MTX), based on the dihydrofolate reductase (dhfr) gene amplification system lead to stable clones. Finally, we investigated growth kinetics, specific productivities, and gene copy numbers (GCN) of these clones and compared the results to the data we obtained for recombinant EpoFc cell lines which were transfected under serum-dependent conditions. By demonstrating that our "next generation" clones show comparable parameters, we are confident that a significant improvement in cell culture technology is possible by switching to protein-free transfection with the advantages of circumventing PFA and its risk of changes in clone performance and reduced expenses, in order to fulfill regulatory demands.

Materials and Methods

CHO Host Cell Line

Serum-dependent dihydrofolate reductase-deficient Chinese hamster ovary cells DUKX-B11, ATCC CRL-9096 (Urlaub and Chasin, 1980) were cultivated in DMEM supplemented with 4 mM L-Glutamine, 10% FCS, and HT (hypoxanthine and thymidine). In order to generate a protein-free host, cells were adapted by stepwise deprivation of FCS to growth in DMEM containing 4 mM L-Glutamine, 0.25% Soya-Peptone, 0.1% Pluronic F-68, HT, and an in-house developed protein-free supplement.

Cells were passaged in a ratio of 1:5 (passage after 3 days) to 1:10 (after 4 days) twice a week. Cultivation was either performed in Nunclon flasks or in spinners in case of suspension cells.

Transfection

Figure 1 gives an overview of the clone families generated by serum-dependent and protein-free transfection.

Plasmids

The plasmids used for transfection were pEpoFc (Fig. 2; obtained from Sandoz[Q1], Austria) containing the cDNA for the human erythropoietin-human immunoglobulin gamma 1 Fc (IgG_1Fc) fusion protein (EpoFc; estimated molecular weight 112 kDa) under the control of the CMV (cytomegalovirus)-promoter and p2_dhfr (Sandoz, Austria), containing the mouse dhfr coding region. Plasmids were purified using the Qiagen[Q2]EndoFree Plasmid Maxi Kit, no linearization was performed prior to transfection.

Serum-Dependent Transfection

Recombinant CHO cell lines which express the fusion protein EpoFc were generated by lipofection. CHO dhfr$^-$

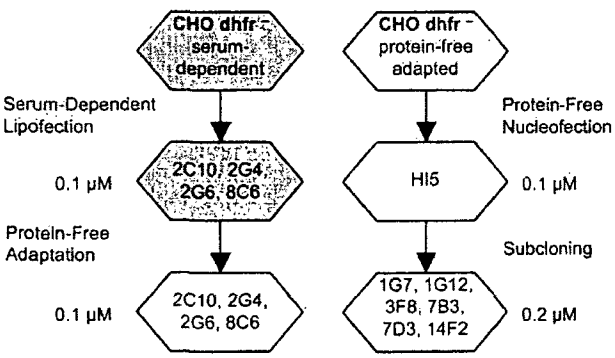

Figure 1. Genealogy of recombinant EpoFc producing cell lines with their respective MTX concentrations: White areas indicate protein-free conditions; gray areas indicate cultivation in serum-containing media.

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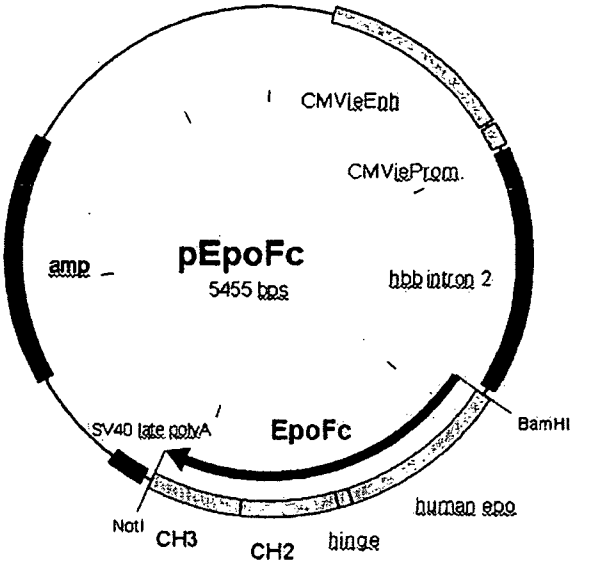

Figure 2. pEpoFc used for transfection of the gene encoding the fusion protein EpoFc. The target gene is under control of the CMV promoter and was ligated into the plasmid after restriction with NotI and BamHI.

cells (30–50% confluency) were cotransfected in 96-well plates with both plasmids (pEpoFc: p2_dhfr = 20:1; 0.2 μg DNA/cm^2 and 0.8 μL Lipofectin/cm^2 (Invitrogen[Q3]), according to the manufacturer's instructions). The cells were incubated in host cell line medium for 24 h before selection medium (host cell line medium without HT, supplemented with 0.05 μM MTX) was applied to the cells. When the first wells became confluent about 3–4 weeks after transfection, selection was continued in the presence of 0.1 μM MTX in 6-well plates and after 6 weeks, clones were expanded into T-flasks. Finally, clones exhibiting highest titers were characterized in four consecutive passages in T25-flasks between seeding (5×10^5 cells per flask) and reaching confluence after 3–4 days. Analysis of cell number and EpoFc titer in the supernatant took place at the end of each experiment.

Clones showing high specific productivities were then adapted over 1 week to protein-free cultivation conditions in DMEM/HAMs-F12 1:1 mixture, supplemented with 4 mM L-Glutamine, 0.25% Soya-Peptone, 0.1% Pluronic F-68, and an in-house developed protein-free supplement. After 2 weeks (splitting ratio 1:3 every 3 days), four clones with best productivities and growth rates (2C10, 2G4, 2G6, and 8C6) were transferred to 125 mL spinner flasks and cultivated for five passages with a selection pressure of 0.1 μM MTX. During that time, the clones were analyzed for their specific productivity in the culture supernatant averaged over three passages. For this purpose, 2×10^5 cells/mL were seeded, and cell concentration as well as product concentration were determined during exponential growth. DNA and RNA were isolated from the cells for genetic analysis in the second and fourth passages.

Protein-Free Nucleofection and Cultivation of Primary Transfectants

For transfection, suspension-adapted dihydrofolate reductase-deficient CHO cells in the exponential growth phase were used (cell density $5–7 \times 10^5$ cells/mL). Cells were cotransfected with the two plasmids pEpoFc and p2_dhfr (pEpoFc: p2_dhfr = 20:1) under protein-free conditions by the Nucleofector™ technology (Amaxa[Q4]), using the cell line Nucleofector™ Kit T. In two transfection experiments (NF1, NF2), the protocol was optimized regarding DNA amount, cell number, and by applying the two pulse programs developed for CHO cells, H-14 (resulting in a high cell survival) and U23 (leading to a high transfection efficiency) as described in the Nucleofector™ manual. In NF1, 8×10^5 and 5×10^6 cells/mL were transfected with 1 and 5 μg DNA, whereas in NF2, additionally, 10 and 20 μg of DNA were used.

The required volume of host cell suspension was centrifuged at 200*g* for 10 min. The supernatant was discarded and the pellet was resuspended in Nucleofector™ Solution. Finally, the cell suspension was mixed with the desired amount of plasmid DNA (pEpoFc: p2_dhfr = 20:1) and pulsed. Afterwards, the cells were transferred into prewarmed protein-free host cell line medium. 24 h later, the medium was changed to a protein-free selection medium (protein-free host cell line medium without HT) and cells were split into two groups and transferred to 12-well plates. As preliminary experiments have shown that adaptation to MTX immediately after transfection was not successful for all clones, one part of the transfected cell pool remained without MTX and the other half of cells was selected with 0.05 μM MTX. During the selection period of 2–4 weeks in 6-well plates, the medium was replaced twice a week. After this selection period, adaptation to 0.05 μM MTX was completed and growth became evident. After three passages (1:3 every 3–4 days) in 6-well plates, the cell pools were transferred to T25-flasks, where cell aggregation was minimized by 1:3 dilutions every 3–4 days and after 2 weeks (in total about 8 weeks after nucleofection), batch experiments were performed in order to investigate the influence of the transfection parameters on the productivity of the cell pools: cells were seeded at a concentration of 2×10^5 cells/mL and specific productivities were determined in the exponential growth phase.

Eight pools with best specific productivities and good growth properties were chosen for further characterization in 125 mL spinner flasks. For this purpose, the MTX pressure was increased to 0.1 μM and 4 weeks afterwards, we performed batch experiments with an initial cell number of 1×10^5 cells/mL to determine growth rate and productivities. Based on these results, one cell pool, HI5, was chosen for subcloning.

Subcloning of Primary Protein-Free Transfectants

Stability of the pool HI5 was determined over 26 passages, representing more than 3 months of cultivation in spinner flasks (seeding density:1×10^5 cells/mL, passaging every 3–4 days). Afterwards, subcloning experiments of HI5 in 96-well plates were started. As the cloning efficiency was rather low in freshly prepared selection medium, the selection medium was supplemented with sterile culture supernatant from the initial pool HI5, harvested at a cell concentration of 4×10^5 cells/mL, 3 days after seeding at a concentration of 1×10^5 cells/mL. Additionally, the optimal cell concentration was determined by a dilution series ranging from 16 cells to 1 cell/well. Finally, subcloning was done at a concentration of 2.5, 5, 7.5, and 15 cells/well with 50% of culture supernatant in the selection medium containing 0.1 μM MTX.

Three to four weeks after subcloning, 44 wells with single-cell clones exhibiting high product titers (5% of all clones) were expanded into 24-well plates, where the MTX concentration was raised to 0.2 μM. After transfer in T25-flasks, clones were again characterized for product titer and based on these results, the number of clones was reduced to 18. Finally, 10 clones with specific productivities >16 pg·cells^{-1}·day^{-1} were chosen for cultivation in spinner flasks. Cell banking was performed in the fourth passage of spinner cultivation (approximately 10 passages after subcloning) and after revitalization from this cell bank, six subclones (HI5/1G7, HI5/1G12, HI5/3F8, HI5/7B3, HI5/7D3, HI5/14F2) were cultivated in suspension for 15 passages in 125 mL spinner flasks with a selection pressure of 0.2 μM MTX. During that time, the subclones were analyzed for their specific productivity in the culture supernatant starting with an initial cell concentration of $1–2 \times 10^5$ cells/mL. Cell concentration as well as product concentration were determined during exponential growth. Results of 15 passages were averaged. Material for genetic analyses was taken in the fifth and seventh passages (17 and 19 passages after subcloning, respectively).

Determination of Growth Rate and Specific Productivity

For determination of growth rate and specific productivity, cell concentration was determined for adherent cells in the Hemocytometer, for suspension, cells analysis was performed in a Multisizer™ 3 Coulter Counter® (Beckman Coulter[Q5]).The secreted EpoFc concentration was quantified in an ELISA. Goat anti-human IgG γ-chain specific (1:500 in 0.1 M $NaHCO_3$; pH 9.6; Sigma[Q6]) was coated overnight on 96-well plates (NUNCLON™). After three washings, serial dilutions (in 1% BSA containing Tween-PBS) of samples and standards were incubated for 1 h at room temperature. After additional washing, detection was accomplished through incubation with HRP (horse radish peroxidase) goat anti-human IgG γ-chain (1:1,000 1% BSA in TPBS; Zymed[Q7]) for 1 h at room temperature and staining with OPD. Affinity purified EpoFc was used as a standard, starting with 0.2 μg/mL.

Specific productivity was then calculated, based on the following equations:

$$\text{Specific productivity}: q_P = \frac{\Delta P}{\text{CCD}} \cdot 1000000 \quad [\text{pg} \cdot \text{cells}^{-1} \cdot \text{day}^{-1}]$$

Cumulative Cell Days (CCD) represent the accumulation of cells during the process and ΔP represent the amount of product generated between t_i and t_{i+1} ($\Delta P = P_{i+1} - P_i$).

Cumulative Cell Days :

$$\text{CCD} = \int (X \cdot dt) = \sum_{i=1}^{n} \frac{(X_{i+1} - X_i) \cdot (t_{i+1} - t_i)}{(\ln(X_{i+1}) - \ln(X_i))} [\text{cells} \cdot \text{day}]$$

X_{i+1} and X_i are cell numbers at the times t_{i+1} and t_i, $(t_{i+1} - t_i)$ is the time interval between sampling.

Low viability as well as cell aggregation (therefore, viability determination by hemocytometer was not possible) of the protein-free transfected cell pools was a major problem during our studies. Therefore, for determination of specific productivity, only particles in the size of G1–G2 phase of the cell cycle (determined for the host cell line) were counted by the Multisizer™ in order to eliminate debris and apoptotic cells.

Investigation of Genetic Parameters

Isolation of genomic DNA was performed by using the QIAamp® DNA Blood Mini Kit (Qiagen) according to the manufacturer's instructions. For Southern blot analysis, genomic DNA was digested with the pEpoFc single cutter BamHI (the EpoFc gene was inserted into the BamHI/NotI opened vector). BamHI digested genomic DNA of each clone (1.6 μg in case of serum-dependent clones after PFA, 3.9 μg of HI5 subclones) as well as 0.01, 0.1, and 1 ng of BamHI digested plasmid DNA standards (pEpoFc; 1 ng = 1.67×10^8 copies) were separated on a 1% agarose gel and transferred to the positively charged nylon membrane, according to the Roche Manual (DIG Application Manual for Filter Hybridization). After prehybridization (7% SDS, 50% Formamide, 5 × SSC, 2% Blocking Reagent, 50 mM Sodium-phosphate pH 7.0, 0.1% *N*-Lauroylsarcosine), the membrane was incubated in a hybridization buffer which contained 10 ng/mL of the denatured probe (a DIG-labeled Fc specific amplicon, 581 bp in length) for at least 12 h at 42°C. Washing and detection were performed as described by Roche using Anti-Digoxigenin-AP Fab fragments (Roche[Q8]) and CPD-Star® (Tropix[Q9]). Visualization was done on the LumiImager™ (Roche).

For Northern blot, RNA was prepared with TRIzol (Invitrogen) from 5×10^6 cells according to the manufacturers instructions. One microliter of RNA (1–3.2 ng/μL) was separated on a Formamide/ Formaldehyde agarose gel according to Maniatis[Q10](1982). The same probes and

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methods were used for hybridization as described for Southern blot.

Six nanograms of genomic DNA (containing 1,800 copies of the genome based on the weight of 3.2 pg DNA per genome) were analyzed by Taqman quantitative PCR in the Rotor-Gene 2000 (Corbett Research[Q11]) using the iQ Supermix (Biorad[Q12]). The plasmid standard pEpoFc (5,455 bps, stock solutions of 1×10^8 copies/μL stored at −20°C) was used in a range from 3×10^6 to 3×10^3 copies in a 1:10 dilution series analyzed in duplicates. In case of theoretically 10 copies of target gene per cell, the minimum amount of genomic DNA template was 1 ng (representing 300 genomes of the CHO cell line) that could be detected in this method.

For determination of mRNA copy number by quantitative PCR, RNA was isolated from 2.5×10^6 cells using the RNeasy Mini Kit (Qiagen). After DNase treatment and reverse transcription, cDNA was purified by using the QIAquick PCR Purification Kit (Qiagen). Concentration was quantified spectrophotometrically at 260 nm. cDNA was adjusted to a concentration of 20 ng/μL and stored at −20°C. cDNA (6 ng) was analyzed as described for genomic DNA. Calculation of the transcript copy was based on the quotient $q_{cDNA/RNA}$ obtained from the amounts of reversely transcribed RNA and resulting cDNA. Finally, the initial cell number analyzed in quantitative PCR was recalculated by the average amount of 12.2 pg of total RNA per cell.

Results

Determination of Optimal Parameters for Protein-Free Nucleofection

Figure 3 shows the influence of each investigated parameter (DNA amount, nucleofection program, and cell number) on the specific productivity of the cell pools generated in the 24 nucleofection experiments. The expression titer of the transfectants was determined 8 weeks after transfection where the viability was higher than 50%. As specific productivities from the same nucleofection assay but with a different time of adaptation to MTX showed comparable results, only one value for each experiment is represented in Figure 3. In batch experiments of both nucleofections, specific productivity was ranging between 0 and 3.4 $pg \cdot cells^{-1} \cdot day^{-1}$, although results have to be regarded with caution as some cell pools exhibited low viability. In general, best results were obtained with 5 μg DNA (Fig. 3A and B), with only one exception, where the yield of the transfection was poor (Fig. 3B, 8×10^5 cells). The average specific productivity was higher in experiments using program H-14, which can be explained by the higher viability of the transfection pool due to the less stringent nucleofection parameters. For the amount of DNA and the nucleofection program, we observed the same optimal parameters in both nucleofection experiments. Regarding in contrast, the cell number in NF1 best results were obtained for 8×10^5 cells, while in NF2 transfection of 5×10^6 cells was more successful. The result of the second nucleofection is more realistic, as a sixfold higher number of cells leads to a higher number of positive transfectants. The experiment using 20 μg of DNA for transfection with the program U-23 was not successful.

Eight cell pools which showed productivities in the range of 1–10 $pg \cdot cells^{-1} \cdot day^{-1}$ were selected for further characterization in spinner flasks. During that time, viability could be increased to 90%—whereas at the beginning of spinner cultivation, several cell pools exhibited only 50% viability. Growth rates were in the range of 0.25–0.5 d^{-1}, specific productivities were ranging from 0.6 to 11 $pg \cdot cells^{-1} \cdot day^{-1}$. Cell pool HI5, which showed the

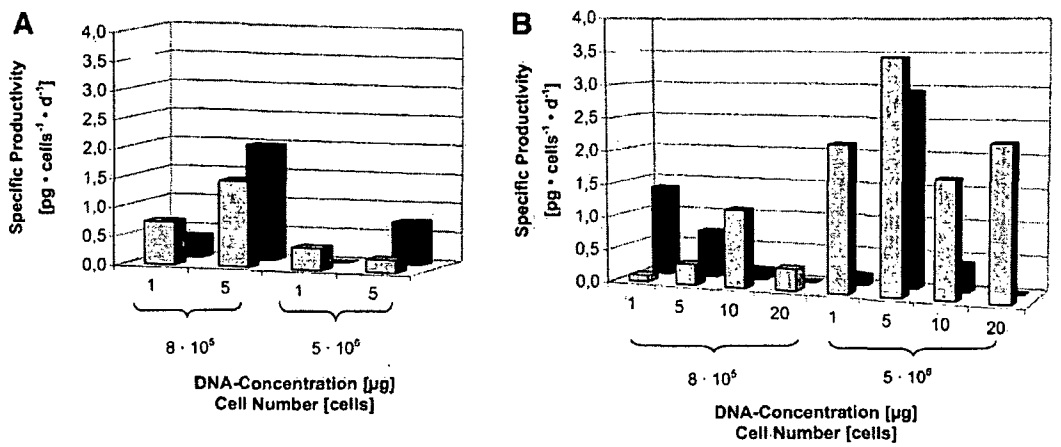

Figure 3. Influence of the different nucleofection parameters on the specific productivity after investigation in batch experiments, which were performed 8 weeks after transfection. On the left side (A) results of NF1, on the right side (B) data from NF2 are shown. Gray bars indicate data from nucleofections performed with program H-14, black bars show data from transfection with program U-23. In both experiments, 8×10^5 and 5×10^6 cells were tested, DNA varied from 1 to 20 μg.

Table I. *Cloning efficiencies of the subcloning experiments with selection medium, supplemented with different amounts of culture supernatant of HI5.*

Experiment	Number of plates	Cell concentration (cells/well)	Culture supernatant (%)	Cloning efficiency (%)
1	18	100–1	0	14–0
2	3	Serial dilutions	12.5	0
			25	20
		16–1	50	65
3	3	Serial dilutions	12.5	15
			25	45
		16–1	50	55
4	15	2.5	50	26
		5		46
		7.5		89
		15		99

Successful subcloning was achieved by using 50% of culture supernatant in the selection medium. The cloning efficiencies of the dilution plates were calculated by averaging the different seedings.

highest amount of productivity, was chosen for subcloning. This cell pool was cultivated over 30 passages and secreted about 11 pg · cells^{-1} · day^{-1} to the medium.

Protein-Free Transfectants

The different subcloning experiments are shown in Table I. Due to the low cloning efficiency we observed in the first experiment when using protein-free selection medium only, subcloning with different amounts (12.5, 25, and 50%) of culture supernatant of HI5 in the selection medium was performed. For this purpose, HI5 was seeded with a starting cell density of 1×10^5 cells/mL, the culture supernatant was then taken 3 days later at a cell density of approximately 4×10^5 cells/mL. Cloning efficiency was tested in the range of 1–16 cells/well. Highest efficiencies were observed in experiments 2 and 3 when using 50% of culture supernatant. In order to obtain single-cell clones, cells were finally seeded at a concentration of 2.5, 5, 7.5, and 15 cells/well according to Table I (experiment 4), resulting in cloning efficiencies of 26, 46, 89, and 99%, respectively.

Growth rates (μ) as well as specific productivities (q_p) of the six subclones (HI5/1G7, HI5/1G12, HI5/3F8, HI5/7B3, HI5/7D3, HI5/14F2) were determined over 15 passages in spinner flasks. Both parameters were stable over this period, the average results are shown in Figure 4. While the subclones showed equal growth rates of about 0.50 d^{-1} (the protein-free host exhibited a growth rate of about 0.53 d^{-1}), productivities were in the range of 11–27 pg · cells^{-1} · day^{-1}, in comparison to the initial cell pool, HI5, that exhibited about 11 pg · cells^{-1} · day^{-1}. Therefore, except from subclone HI5/1G7, an increase in q_p in a range of 1.5- to 2.5-fold could be observed, which can be explained by the combination of subcloning and increase of MTX pressure.

Serum-Dependent Transfectants

Table II lists the specific productivities of the investigated clones before and after PFA. 2G4 was the only clone that

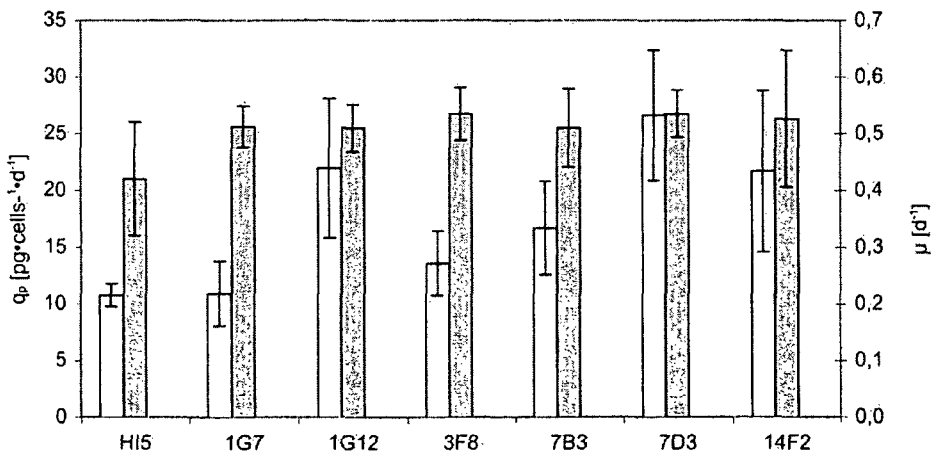

Figure 4. Characterization of the six investigated subclones of HI5 in spinner cultivation. Data of specific productivity are shown in white columns, gray columns represent growth rates. Compared to the cell pool HI5, specific productivity could be increased from 11 to 27 pg · cells^{-1} · day^{-1}.

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Table II. Changes in specific productivities of the clones after protein-free adaptation (PFA).

Clone	q_p before PFA ($pg \cdot cells^{-1} \cdot d^{-1}$)	q_p after PFA ($pg \cdot cells^{-1} \cdot d^{-1}$)
2C10	57	27
2G4	13	19
2G6	43	31
8C6	15	5

could increase the specific productivity in protein-free medium. The other three clones maintained between 35 and 70% of their initial productivity. Growth rates of the clones were in a range of 0.50–0.64 d^{-1} during serum-dependent cultivation. After PFA, growth rates increased to the range of 0.50–0.80 d^{-1}.

Stability of the clones was determined over 15 passages of protein-free cultivation in spinner flasks, and no significant changes in productivities were observed during this long-term culture.

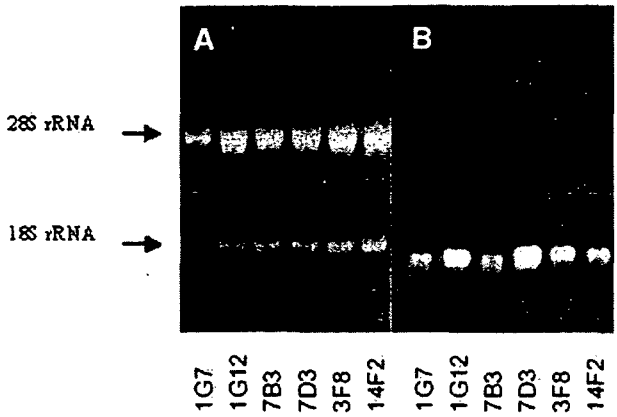

Figure 6. RNA-Gel (A) and Northern blot (B) of the six HI5 subclones showing an intact specific transcript of approximately 1.3 kb in length below the 18S rRNA (assumed length 1.9 kb).

Genetic Characterization of Clones

Southern hybridization of BamHI digested genomic DNA with the EpoFc probe gave the same restriction pattern for clones 2C10 and 2G6, as well as for 2G4 and 8C6, indicating equal integration sites (Fig. 5A); concerning the protein-free transfectants, we observed the equal restriction pattern for all six subclones (Fig. 5B). Therefore, we assume that the initial cell pool, HI5, was already very homogenous when subcloning was performed.

As the BamHI restriction site is at the 5′ site of EpoFc in the pEpoFc, bands showing equal size but different intensity on the Southern blot result from different GCN.

Northern blot analysis of the protein-free subclones revealed intact mRNA (see Fig. 6). EpoFc mRNA (1.3 kb in length) appears in the Northern blot (Fig. 6B) below the 18S rRNA (assumed length 1.9 kb). Same results were observed for the serum-dependent transfectants (data not shown).

Figure 7 shows the GCN as well as the amount of transcript (mRNA) of the two clone families in a logarithmic scale. The GCN for the protein-free adapted serum-dependent transfectants was in a range of 3–25 copies/cell, the subclones generated by protein-free transfection had only 4–13 copies of the target gene integrated into their genome (Fig. 7). The specific mRNA copy number of the serum-dependent transfectants was about 2,000–6,000

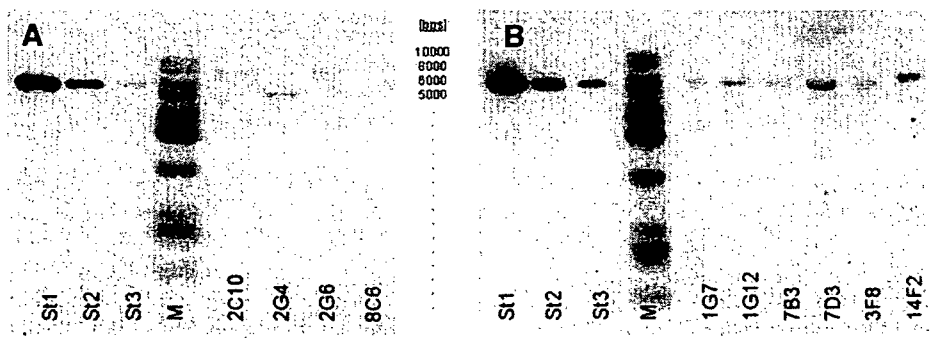

Figure 5. Southern blot after digestion of genomic DNA: St1, St2, St3: linearized pEpoFc (2×10^8, 2×10^7, 2×10^6 copies; 5,455 bps); M: Gene Ruler DNA Ladder Mix (MBI Fermentas); Further lanes show genomic DNA of the clones (A: serum-dependent transfectants after protein-free adaptation (PFA), B: subclones of protein-free transfected HI5). Concerning clone 8C6 (A), the band appeared after longer exposition at the same height as the 2G4 band (approximately 5,000 bps). In the middle of both figures, the lengths of the first four marker bands are listed.

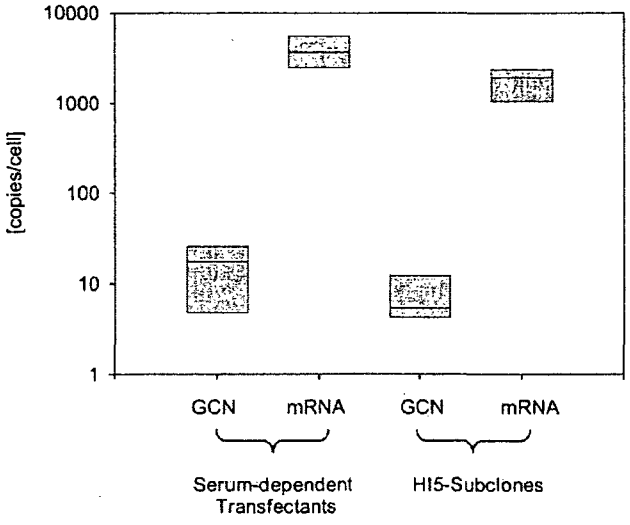

Figure 7. Box-Plot presentation of genetic parameters (GCN and mRNA level) of the two clone families, the protein-free adapted transfectants on the left side, the HI5 subclones on the right side. Boundaries of the boxes present 25th and 75th percentiles, the lines within the boxes mark the median. As it can be observed, the GCN of the serum-dependent transfectants are about two times higher than the values from the protein-free transfectants.

copies/cell, which is about 2.4-fold higher than the amount of transcript in HI5 subclones.

Discussion

In this study, we describe a protein-free transfection protocol which results in stable cell lines expressing the fusion protein EpoFc. The optimal amount of 5 μg of DNA per transfection experiment was in the range as reported by Kuchenbecker et al. (2005). When using Nucleofector™ Kit T (designed for CHO), two different pulse programs are suitable for transfection. Program H-14 is less harmful to the cells and, therefore, results in a higher cell survival, but transfection is less efficient. By using program U-23, higher efficiency but lower viability after transfection is obtained due to stronger pulse parameters; we achieved higher specific productivities of primary transfectants by using program H-14. Nevertheless, still a large amount of dead cells is present in the culture after nucleofection. Therefore, we cultivated the cell pools with a very careful handling (only exchanging the culture supernatant partly with fresh medium, instead of centrifugation in the first weeks after transfection) until growth became evident about 3 weeks after nucleofection. Furthermore, by adaptation to MTX not immediately after transfection, we could finally obtain growing cell pools from nearly all transfections even if a lot of cell debris could not be removed efficiently. In contrast, in adherent systems that are mainly based on serum, dead cells detach and can be easily removed by medium replacement.

As far as adaptation to MTX was concerned, no significant differences in specific productivities were observed for those experiments which differed only in the time of MTX adaptation. In case of protein-free transfection of EpoFc, immediate adaptation to MTX was possible when using program H-14, although in some transfections of other exogenous genes immediate adaptation to MTX was not successful. Therefore, we suggest that this parameter has to be investigated separately for each recombinant cell line.

Figure 8 gives an overview of the data we obtained for the different clone families. Genetic parameters were only determined for the protein-free adapted serum-dependent transfectants as well as for the subclones of HI5 (Fig. 8B and C). For the protein-free adapted clones as well as the

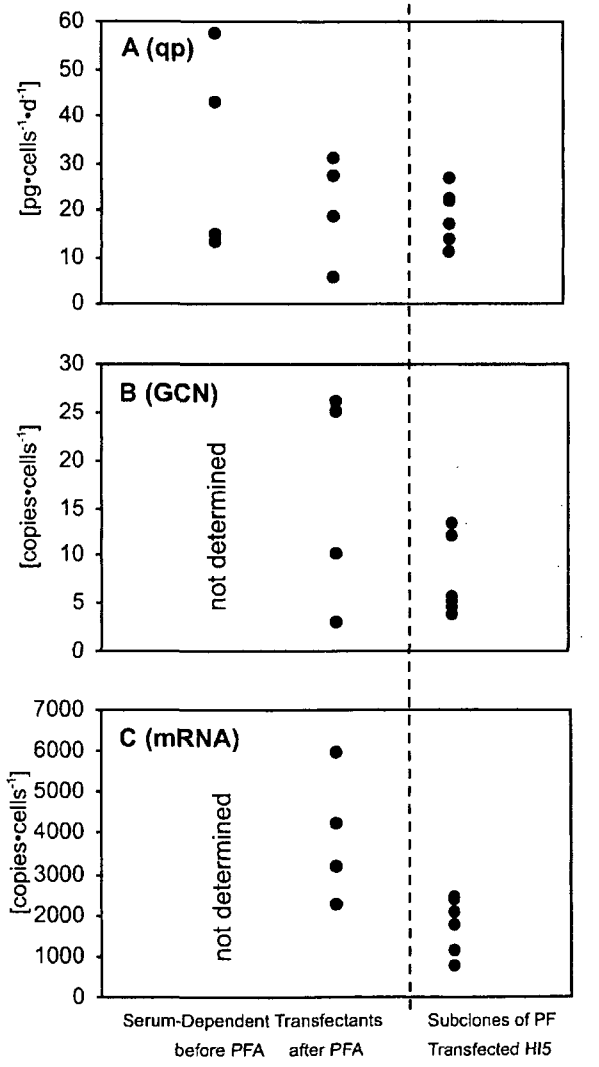

Figure 8. Specific productivity (A), GCN (B), and mRNA level (C) of the serum-dependent transfectants before and after PFA as well as of the subclones of protein-free (PF) transfected HI5.

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protein-free transfected subclones, comparable specific productivities in the range of 5–27 pg · cells^{-1} · day^{-1} were obtained (see Fig. 8A) due to a drop of the productivity after PFA of the recombinant cell lines. However, the slightly higher average q_p of the protein-free adapted clones might result from the twofold higher mRNA levels and GCN (Fig. 8B and C). Clone 2G6 exhibited about 31 pg · cells^{-1} · day^{-1} with a GCN of 25 and a mRNA level of 6,000 copies/cell—in contrast, the best producing subclone, HI5/7D3, showed a specific productivity of 27 pg · cells^{-1} · day^{-1} with a GCN of 12 and a mRNA level of 2,400 copies/cell. Thus, the specific productivity did not increase in the same ratio like the gene copy number and also the 2.5-fold higher amount of transcript (Fig. 8C) in the best producing protein-free adapted clone does not yield a comparable improvement in specific productivity. Since the specific productivity remarkably decreases during PFA of serum-dependent recombinant clones, we either assume a less efficient translation machinery as mRNA levels remained constant (data not shown) or problems during PFA.

The integration locus does not seem to remarkably affect q_p when comparing the different clone families, as the same range of q_p was obtained from cell lines showing different restriction patterns in Southern blot analysis: The restriction fragment of clones 2C10 and 2G6 (27–31 pg · cells^{-1} · day^{-1}) were about 8,000 bps in length, whereas the bands of the HI5 subclones (11–27 pg · cells^{-1} · day^{-1}) appeared at 6,000 bps. When comparing GCN determined by quantitative PCR to band intensities in the Southern blot, for clones 2C10, 2G4, and 2G6, equal intensities are observed (26, 10, 25 copies/cell determined by qPCR). Clone 8C6 had three copies of the target integrated into the genome, in Southern blot, the restriction fragment was only detected after longer exposition. Looking at the protein-free transfectants, highest GCN were achieved for clones HI5/7D3 and HI5/14F2 (12 and 13 copies/cell, respectively); in Southern blot, highest intensities were observed for these two clones, too. Furthermore, we could show that the serum-dependent transfectants represent stable clones by controlling q_p and μ over several months. Thus, we preclude that the reduction of specific productivity after PFA is deduced from a reduction of the GCN.

Seven-day batch titers of the subclones of the protein-free transfectants were in the range of 56–120 μg/mL EpoFc, which is comparable to the titers of the protein-free adapted clones that were in the range of 12–110 μg/mL EpoFc. Therefore, protein-free transfection is a far more efficient strategy for recombinant clone development. In addition, we have shown in Figure 8A and Table II that changes in clone properties after PFA are also evident for specific productivity. With protein-free generated clones, the problem of clonal changes and instability is circumvented and relevant parameters allowing the prediction of clonal performance can be applied earlier in clonal development. In conclusion, we could successfully demonstrate that the basic requirements for transfection of protein-free adapted host cells, namely, comparable production rates as well as product qualities (Schriebl et al., 2006), are achievable by our transfection and screening program.

Nomenclature

Glossary

CHO	Chinese ovary cell
dhfr	dihydrofolate reductase
GCN	gene copy number
MTX	methotrexate
NF	nucleofection
PFA	protein-free adaptation

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Characterisation of recombinant CHO cell lines by investigation of protein productivities and genetic parameters

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Abstract

We have generated a recombinant CHO cell line expressing the fusion protein EpoFc. After selection and screening, protein expression, gene and mRNA copy numbers were analysed in order to gain more information on the influence of genetic parameters on the productivity and stability of production cells. Results from semi-quantitative blot methods were compared to quantitative PCR (qPCR) analyses, whose advantage mainly lies in its higher sensitivity, and the cheaper and faster methodology.

We developed stable and high producing clones with low gene copy numbers, in contrast to other cell lines where multiple steps of methotrexate amplification have lead to hundreds of copies of inserts with the risk of karyotypic instabilities and decreased growth rates that overcome the benefits of increased productivities.

When comparing genetic parameters to productivity, a good correlation of mRNA levels with specific productivity was observed, whereas high gene copy numbers were not always accompanied by high protein expressions. Based on our data derived from a typical example of a cell line development process, genetic parameters are useful tools for the selection of scalable production clones. Nevertheless, a wider range of cell lines has to be investigated in order to implement genetic analyses into a screening process.

Keywords: CHO cells; Quantitative PCR; Taqman; Genetic characterisation; Genetic parameters; Gene copy number

Abbreviations: CHO, Chinese Hamster Ovary; dhfr, dihydrofolate reductase; EpoFc, erythropoietin-huIgG1Fc fusion protein; GCN, gene copy number; MCB, master cell bank; MTX, methotrexate; OPD, orthophenylenediamine; q_p, specific productivity; qPCR, quantitative PCR; RT, reverse transcription

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1. Introduction

Gene amplification techniques are frequently employed for the production of glycoproteins and monoclonal antibodies in recombinant cell technology. In case of the Chinese Hamster Ovary (CHO) cell lines, the dihydrofolate reductase (dhfr) gene amplification system is the most widely used (Alt et al., 1978; Kaufman, 1993), resulting in high productivities as well as high gene copy numbers (GCNs) in the range of some hundred inserts (Kaufman et al., 1985; Kim et al., 1998b, Yoshikawa et al., 2000) after several steps of methotrexate (MTX) amplification. On the other hand, negative effects (like chromosome rearrangements and recombination in response to amplification procedures) of high gene copy numbers on karyotypic stability are reported especially after long time cultivation (Hammil et al., 2000; Michel et al., 1985; Pallavicini et al., 1990; Bacsi et al., 1986; Flintoff et al., 1984). Furthermore, a high gene copy number might have a detrimental effect on the cell growth, which may outweigh any beneficial effect of an enhanced specific productivity (q_p). Besides, analysis of mRNA copy number showed a positive correlation with specific productivity (Kaufman et al., 1985; Gu et al., 1992; Pendse et al., 1992; Kim et al., 1998a). Nevertheless, during long-term cultivation of different subclones of antibody expressing CHO cells no correlation between q_p and GCN and only a weak correlation of q_p and mRNA was observed (Kim et al., 1998b).

Beside the investigation of genetic parameters in order to gain additional criteria for clone selection, knowledge of these data is also required from regulatory authorities: FDA, EMEA and the European Pharmacopoeia suggest analysis of the expression construct for copy number, insertions, deletions and the integration status as well as the number of integration sites in order to evaluate the stability of the cell banks. For characterisation of the master cell bank (MCB) and the end-of-production cells Northern blot and Southern blot are commonly used, although only semi-quantitative information is gained. The rather new method of quantitative PCR (qPCR) has not yet become part of routine analyses.

In our studies we analysed different subclones of a recombinant EpoFc (erythropoietin-huIgG1Fc fusion protein) producing cell line by qPCR and blot methods. Beside the evaluation of these methods we investigated the influence of genetic parameters on the productivity and stability of our subclones. Afterwards we will discuss the use of genetic parameters as criteria for clone selection.

2. Materials and methods

2.1. Recombinant production clones

Recombinant CHO cell lines expressing the fusion protein EpoFc were generated by lipofection of dihydrofolate reductase (dhfr)-deficient, serum-dependent Chinese Hamster Ovary Cells, DUKX-B11, ATCC CRL-9096 (Urlaub et al., 1980) in 96-well plates. The plasmids used for co-transfection were pEpoFc (Fig. 1) containing the cDNA of EpoFc (one molecule of erythropoietin joined to each hinge region of huIgG1Fc) under the control of the CMV (cytomegalovirus)-promoter. The second plasmid was p2_dhfr, containing the mouse dhfr coding region. Circular plasmid DNA was purified using the Qiagen EndoFree Plasmid Maxi Kit and used for transfection. Selection of recombinant cells was performed in the presence of 0.05, and afterwards 0.1 μM MTX. After 18 passages in T25-flasks (Nunclon) 5 primary transfectants (2C10, 2G4, 2G6, 8C6 and 10D9) were adapted to protein-

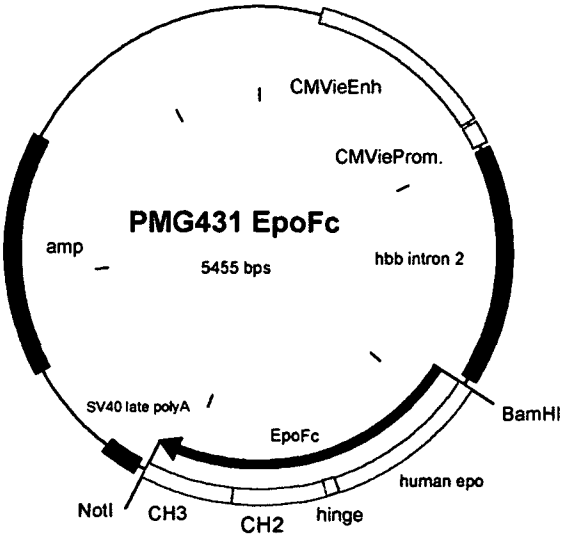

Fig. 1. pEpoFc used for transfection of CHO dhfr DUKX-B11. The target gene is under control of the CMV-promoter and was ligated into the plasmid after restriction with *Bam*HI and *Not*I.

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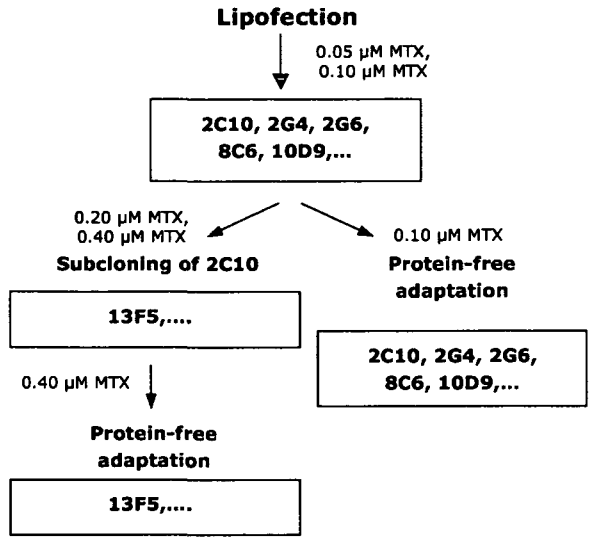

Fig. 2. Genealogy of recombinant EpoFc producing cell lines with their respective MTX-concentrations.

free cultivation conditions in DMEM/HAMs-F12 1:1 containing 4 mM L-glutamine, 0.25% soya–peptone, 0.1% pluronic F-68, HT (hypoxanthine, thymidine), an in-house developed protein-free supplement and 0.1 µM MTX. Finally cell banking was performed 10 passages after protein-free adaptation by nitrogen-cryopreservation in DMSO containing protein-free freezing medium. A serum containing culture of clone 2C10 was adapted to 0.2 and 0.4 µM MTX before subcloning with 7.5 and 15 cells/well. The best producing subclones were then adapted to protein-free cultivation conditions and finally the best performing clone concerning growth and productivity, 2C10/13F5, was chosen for further analysis. The genealogy of the investigated clones is shown in Fig. 2. After revitalisation from the research cell bank, the five primary transfectants and the subclone were cultivated for five passages in 125 ml spinner flasks (Techne) with a selection pressure of 0.1 µM MTX and 0.4 µM MTX in case of 2C10/13F5. Passaging was performed twice a week with a seeding density of 2×10^5 cells/ml in a total volume of 50 ml. Sampling for analyses of product titer was performed during the exponential phase of each passage (at a cell density of $(4–10) \times 10^5$ cells/ml and a viability higher than 95%), material for determination of genetic parameters was taken in passages 2 and 4.

2.2. Determination of specific productivity

For determination of specific productivity, cell concentration was determined in a Multisizer™ 3 Coulter Counter® (Beckman Coulter) and the secreted product concentration was quantified using an ELISA.

The secreted EpoFc concentration was quantified in a sandwich ELISA for the huIgGFc part of the fusion protein. Goat anti-human IgG γ-chain specific (Sigma) was used as catcher antibody, detection was accomplished through incubation with HRP (horse radish peroxidase)–goat anti-human IgG gamma chain (Zymed) and staining with OPD (orthophenylenediamine). Affinity purified EpoFc was used as a standard starting with 0.2 µg/ml.

2.3. Preparation of genomic DNA

2.5×10^6 cells were washed in PBS and nucleic acid isolation was performed using the QIAamp® DNA Blood Mini Kit (Qiagen) according to the manufacturers instructions. The DNA concentration was determined in a BioPhotometer (Eppendorf) at 260 nm. Genomic DNA samples were stored at +4 °C.

2.4. Preparation of RNA and cDNA

For Northern blot, total RNA was prepared with Trizol (Invitrogen) from 5×10^6 cells according to the manufacturers instructions. For quantitative PCR, total RNA was isolated from 2.5×10^6 cells using the RNeasy Mini Kit (Qiagen). Total RNA was eluted in 40 µl and remaining DNA was digested with 5 U DNase I (Promega) prior to reverse transcription (RT). RT was performed at 37 °C for 1 h in a total reaction volume of 25 µl, containing 15.3 µl of eluted RNA, 135 pmol oligo dT(16 mer) (Qiagen), 200 U M-MLV reverse transcriptase (Promega), 12.5 nmol dNTPs (Fermentas) and 40 U ribonuclease inhibitor (Fermentas). Afterwards, cDNA was purified using the QIAquick PCR Purification Kit (Qiagen). Concentration of total RNA as well as of cDNA was quantified spectrophotometrically. cDNA was adjusted to a concentration of 20 ng/µl and stored at −20 °C.

2.5. Southern blot analysis

A DIG-labelled huIgG1Fc specific probe (581 bp) was generated by PCR (PCR DIG Labelling Mix;

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Roche) and purified by QIAquick PCR Purification Kit (Qiagen). Genomic DNA was digested with *Bam*HI cutting at the 5′ site of the EpoFc coding region. 1.5 μg of *Bam*HI-digested genomic DNA of each clone as well as 1, 0.1 and 0.01 ng of *Bam*HI-digested pEpoFc standard (1 ng = 1.7×10^8 copies) were separated on a 1% agarose gel and transferred to the positively charged nylon membrane according to the Roche Manual (DIG Application Manual for Filter Hybridization). After 1 h in prehybridisation buffer (7% SDS, 50% formamide, 5× SSC, 2% blocking reagent and 0.1% *N*-lauroylsarcosine in 50 mM sodium-phosphate pH 7.0), the membrane was incubated in hybridisation buffer additionally containing 10 ng/ml of the denatured probe for 12–18 h at 42 °C. Washing and detection was performed using anti-digoxigenin-AP Fab fragments (Roche) and CPD-Star® (Tropix). Visualisation was done on the LumiImager™ (Roche). Finally, the GCN was determined by the standard curve based on the Boehringer luminescence units (BLU) using the LumiAnalyst 3.0 Software (Filtered Profile, 10%). The calculation of the target gene per cell was based on the size of the hamster genome (3×10^9 bp; Marshall, 2001) that results in 3.3 pg DNA/cell. Thus, 1.5 μg genomic DNA used in Southern blot experiments represent 4.5×10^5 copies of the genome.

2.6. Northern blot analysis

One microliter of the total RNA preparation (analysed in duplicates) and 1 μl of each plasmid standard (1.7×10^8 and 3.3×10^7 copies) were separated on a formamide/formaldehyde agarose gel according to Maniatis et al. (1982). For hybridisation the same probes and methods were used as in Southern blot. The mRNA copy number was determined by the plasmid standard curve as described for Southern blot and was then related to the number of cells from which the analysed amount of RNA was derived.

2.7. Quantitative PCR

Taqman quantitative PCR was performed in the Rotor-Gene 2000 (Corbett Research). The plasmid standard pEpoFc (stock solutions of 1×10^8 copies/μl corresponding to 0.6 ng/μl, stored at −20 °C) was used in the range of 3×10^6 to 3×10^3 copies in a 1:10 dilution series. Six nanograms of genomic DNA and 0.6 ng of cDNA were used as a template. The standard curve was analysed in duplicates, samples in triplicates.

The reaction mix consisted of iQ Supermix (Biorad), 18 pmol of sense and antisense primers and 3 pmol of the fluorogenic probe labelled with 6-carboxyfluorescein (FAM) and the fluorescent quencher dye 6-carboxytetramethylrhodamin (TAMRA). Amplification was done by denaturation at 95 °C for 5 min and afterwards running 45 cycles consisting of 95 °C for 15 s and 60 °C for 1 min. Recording was done at the end of the second step at 495/620 nm, and results were evaluated with the Rotor-Gene Software V 5.0 at a threshold of 0.06.

2.7.1. Validation of qPCR-analyses

For validation, five independent analyses of the standard curve were performed in duplicates. The results were calculated with a statistic program based on an Excel macro. On the basis of these analyses, linear regression (ANOVA), linearity (Mandel), variance homogeneity (*F*-test) and range were statistically evaluated. The logarithm of the copy number was related to the CT-value (cycle number where the fluorescence rises above the defined threshold) in a first order equation (CT = slope log(copy number) + intercept).

2.7.2. Determination of gene copy number

The gene copy number of EpoFc was quantified by analysing 6 ng genomic DNA of each clone (representing 1820 molecules of the genome). Genomic DNA from passages 2 and 4 of each clone was analysed three times and the GCN was averaged from the six results.

2.7.3. Determination of the mRNA copy number

Compared to the GCN, the calculation of the transcript copy number is more complex. In each experiment 0.6 ng cDNA were used as template. Since little is known about the total RNA content per cell, we quantified the RNA isolated from 2.5×10^6 cells and found (comparable to Bustin, 2002) 12.2 ± 3.7 pg total RNA/cell. The initial cell number used for quantitative PCR is influenced by the efficiency of the reverse transcriptase reaction. Therefore we introduced the quotient $q_{cDNA/RNA}$ for back-transformation: initial cell number = analysed cDNA (pg)/($q_{cDNA/RNA}$·12.2 (pg/cell)). RNA from passages 2 and 4 of each clone was analysed three times and the mRNA copy number was averaged from the six results.

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3. Results and discussion

We have generated recombinant EpoFc expressing cell lines after transfection in 96-well plates. Due to this technique we regard our primary transfectants as almost homogeneous clones. As demonstrated in Fig. 2, our primary transfectants as well as the subclone 2C10/13F5 were adapted to protein-free medium and characterised for specific productivity and genetic parameters.

3.1. Determination of specific productivity

Specific productivities of the different clones cultivated in spinner flasks were averaged over five passages: highest titers were reached by the subclone 2C10/13F5 ($46.2 \pm 3.6\,pg\,cell^{-1}\,d^{-1}$), followed by 2G6 ($30.6 \pm 1.5\,pg\,cell^{-1}\,d^{-1}$) and 2C10 ($26.8 \pm 2.2\,pg\,cell^{-1}\,d^{-1}$). Lower titers were obtained for clone 2G4 ($18.6 \pm 1.3\,pg\,cell^{-1}\,d^{-1}$) and 8C6 ($5.5 \pm 1.3\,pg\,cell^{-1}\,d^{-1}$), while clone 10D9 showed poor productivity ($1.6 \pm 0.5\,pg\,cell^{-1}\,d^{-1}$).

3.2. Restriction analysis and quantification of gene copy number by Southern blot

Fig. 3 shows that hybridisation of *Bam*HI-digested genomic DNA with the EpoFc probe revealed three different patterns: a single band with similar electrophoretic mobility was obtained for clone 2C10, 2G6 and 2C10/13F5 (Fig. 3, lanes 5, 7 and 10). Also 2G4 and 8C6 (Fig. 3, lanes 6 and 8) showed a smaller sized single band with a mobility similar to each other. Amplification of the same region surrounding EpoFc might lead to similar restriction patterns in different clones; in addition, we could demonstrate that 2C10, 2G6 and 2C10/13F5 showed a single fluorescence hybridisation signal on the large arm of the same acrocentric chromosome near the telomeric region (Lattenmayer et al., in press). In case of 2C10, 2G6 and 2C10/13F5 the hybridised fragment appears higher than the linearised vector while in case of 2G4 and 8C6 it is lower, which might result from incomplete integration of the pEpoFc shuttle vector. Concerning clone 10D9 (Fig. 3, lane 9), the rather complex pattern of restriction with three specific bands indicates either multiple integration sites (also observed for other clones by Park et al., 2000) or might be due to genetic rearrangement in the genome after adaptation to protein-free conditions, as proposed by Kim et al. (1998a).

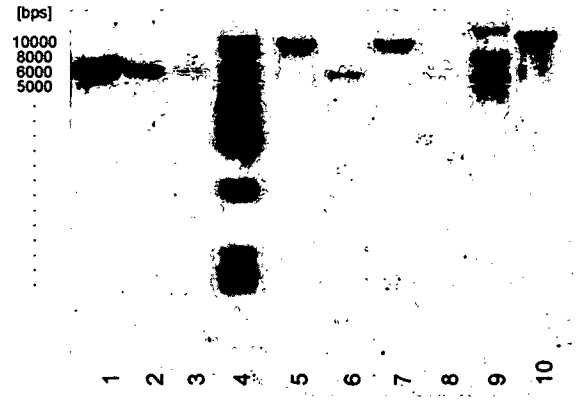

Fig. 3. Southern blot: the size of the first four marker bands is shown on the left side. (1–3) Linearised pEpoFc, 5455 bp (1.7×10^8, 1.7×10^7 and 1.7×10^6 copies); (4) λ DNA/*Eco*RI + *Hin*dIII; (5–10) 1.5 μg of *Bam*HI-digested genomic DNA from clones 2C10, 2G4, 2G6, 8C6, 10D9, 2C10/13F5. 2C10, 2G6 and the subclone 2C10/13F5 showed similar hybridisation signals and so did 2G4 and 8C6 (only very weak signal). Hybridisation of 10D9 genomic DNA resulted in three EpoFc specific fragments.

Furthermore, strange restriction patterns (like 10D9) or hybridised fragments larger than the linearised vector (e.g. 2C10, 2G6 and 2C10/13F5) might occur due to the formation of concatamers: After transfection, the supercoiled plasmid DNA molecules might be converted into linear form by exo- and endonucleases (Finn et al., 1989). Nuclear ligases then join individual plasmid molecules to concatamers of undefined size and structure which are integrated into the host genome (like single plasmid molecules) by non-homologous recombination (Perucho et al., 1980). In order to get more information on the insertion locus detailed investigations have to be done by sequencing the surrounding locus.

The calculated number of target genes detected in Southern blot was divided by the number of loaded genomes resulting in 1–117 copies of EpoFc per cell (see Table 1). The primary transfectants 2C10, 2G4, 2G6 and 8C6 showed a rather low gene copy number (less than 10 copies/cell), therefore we conclude that the observed different restriction patterns might result from different integration sites. Furthermore, the low gene copy numbers might either result from gene amplification at the rather low level of methotrexate pressure or from ligation of plasmid DNA molecules

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Table 1
Quantification of blot analyses resulting in gene and mRNA copy number: the GCN was ranging between 1 and 117, whereas the mRNA copy number was between 240 and 5900 copies/cell

Clone	GCN (Southern blot)		mRNA copy number (Northern blot)		
	Copies/cell	S.D. (%)	Quantified transcript (copies)	RNA loaded (μg)	Copies/cell
2C10	4	111	3.9E+08	1.7	2900
2G4	1	43	4.0E+08	2.1	2400
2G6	2	98	4.3E+08	1.7	3200
8C6	1	0	1.0E+08	1.9	690
10D9	117	21	5.1E+07	2.7	240
2C10/13F5	18	81	7.9E+08	1.7	5900

prior to co-integration at a single site (Wurm, 2004). In contrast, analysis of 10D9 revealed a rather high GCN with 117 copies of EpoFc per cell. In case of the subclone 2C10/13F5 we found a four- to five-fold increase in GCN that can be explained by a four-fold higher MTX concentration in combination with subcloning and screening.

3.3. Determination of mRNA-levels by Northern blot

The Northern blot (see Fig. 4) shows the integrity of specific mRNA for the six recombinant production clones with identical electrophoretic mobility and appeared at the same height as the 18S rRNA due to co-migration. No degraded transcript was observed.

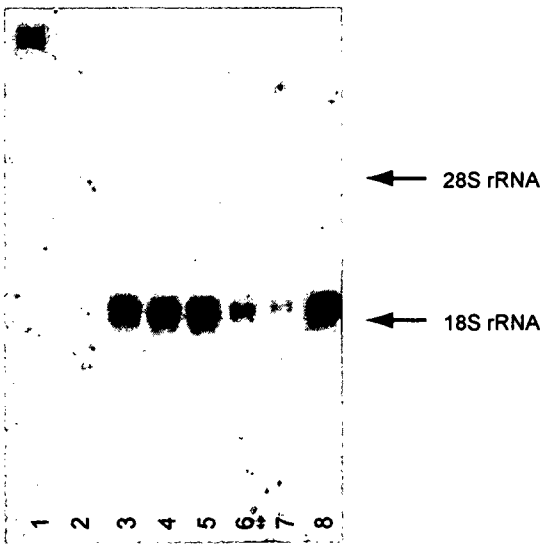

Fig. 4. Northern blot: (1 and 2) linearised pEpoFc (1.7×10^8 and 3.3×10^7 copies); (3–8) 1.7–2.7 ng total RNA (for comparison see Table 1) from clones 2C10, 2G4, 2G6, 8C6, 10D9, 2C10/13F5 hybridised with an EpoFc specific probe. Undegraded transcript of similar size was evident for all investigated transfectants and subclones.

The range of 1.7–2.7 μg of total RNA loaded on the blot represents $(1.4–2.2) \times 10^6$ cells based on the total RNA content of 12.2 pg/cell. The copy numbers of the specific mRNA were calculated via plasmid standards (1.7×10^8 and 3.3×10^7 copies) and divided by the number of cells used for each experiment and are summarised in Table 1. The highest specific mRNA copy number (5900 copies) was achieved by the subclone 2C10/13F5, comparable values were obtained for 2C10, 2G4 and 2G6 ranging from 2400 to 3200 specific RNA copies per cell whereas clone 8C6 and 10D9 showed the lowest transcript copy numbers in a range of a few hundred copies of specific EpoFc mRNA per cell.

3.4. Quantitative PCR

3.4.1. Determination of gene copy number

The absolute amount of EpoFc was analysed in samples containing 6 ng of genomic DNA (representing 1820 copies of the genome) and resulted in the range of 5000–100,000 copies per sample of the investigated clones, thus enabling quantification of gene copy numbers even of cell lines without gene amplification. For example, 3 copies of EpoFc per cell result in 5000 copies per 6 ng, that is quantified by our standard curve ranging from 3×10^6 to 3×10^3 copies. Gene copy numbers per cell are presented in Table 2 with standard deviations in the range of 12–37% except only 5% of deviation for the clone 10D9.

3.4.2. Determination of the mRNA copy number

Two different methods are commonly used for the determination of the specific mRNA copy num-

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Table 2
Quantification of qPCR analyses resulting in gene and mRNA copy number as well as specific productivities: the GCN was ranging between 3 and 140, whereas the mRNA copy number was between 330 and 10,300 copies/cell

Clone	GCN (qPCR)		mRNA copy number (qPCR)		Specific productivity	
	Copies/cell	S.D. (%)	Copies/cell	S.D. (%)	q_p (pg cell^{-1} d^{-1})	S.D. (%)
2C10	26	19	3,100	12	27	8
2G4	10	30	4,200	27	19	7
2G6	25	12	5,900	42	31	5
8C6	3	37	2,200	34	6	25
10D9	140	5	330	10	2	29
2C10/13F5	61	28	10,300	44	46	8

The specific productivity was between 2 and 46 pg cell^{-1} d^{-1}.

ber: one method is the normalisation of the mRNA copy number with a housekeeping gene. The problems with this method do not only arise from the imprecision of quantification of the additional gene but also from the variability of the transcription status of the housekeeping gene. In particular the commonly used glyceraldehyde-3-phosphate dehydrogenase contributes to different cellular functions (Zhu et al., 2001; Sirover, 1999; Tatton et al., 2001; Goidin et al., 2001; Suzuki et al., 2000), and also beta-actin was shown to have different mRNA-levels in murine fibroblasts and human cell lines (Lupberger et al., 2002; Schmittgen et al., 2000) leading to imprecision in calculations of the target gene. The second method that we have used is the absolute quantification of cDNA. We determined the amount of RNA used for transcription, measured the cDNA concentration after reverse transcription and purification and used a constant amount of cDNA as PCR template.

For example, we transcribed 4.4 ng of total RNA of clone 2C10 and obtained 1.4 ng cDNA resulting in a $q_{cDNA/RNA}$ of 0.31. In qPCR we analysed 0.6 ng of cDNA template representing 1.9 ng of initial total RNA (0.6 ng divided by 0.31). This corresponds to 158 cells that were analysed (based on 12.2 pg RNA/cell). Finally we divided the transcript copy number obtained in qPCR by the number of cells that was analysed and summarised all results in Table 2. Clone 10D9 displays the lowest specific mRNA-level, clones 2C10, 2G4, 2G6 and 8C6 were ranging from 2200 to 5900 copies of target mRNA per cell. The subclone 2C10/13F5 showed a 3.5-fold increase in EpoFc transcript copy number compared to its mother clone.

3.5. Comparison of blot analysis to quantitative PCR

Fig. 5 shows the GCN of EpoFc of the six clones obtained after analysis by Southern blot and quantitative PCR. The results of the GCN are shown on a logarithmic scale with standard deviations from four experiments in case of Southern blots and six experiments in case of qPCR.

Results of qPCR were up to 12.5-fold higher compared to Southern blot analyses, with highest differences in the range of low gene copy numbers. However, the GCN of clone 10D9 displaying more than 100 copies/cell determined by qPCR was only 1.2-fold higher than the result of Southern blot analysis. As 1.5 μg of genomic DNA were analysed in Southern hybridisation compared to 6 ng of DNA template in

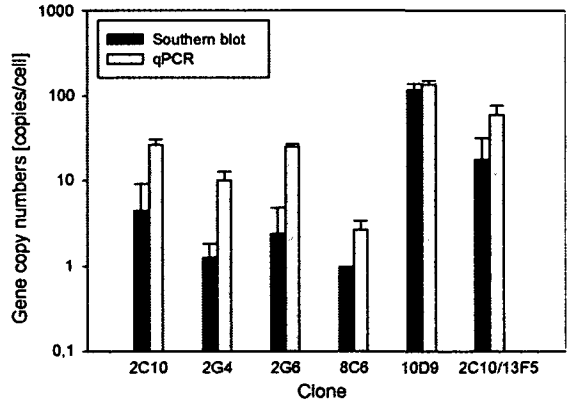

Fig. 5. Gene copy numbers determined by qPCR are higher than in Southern blot. Maximum GCN was evident for clone 10D9 in both methods, while clone 8C6 showed the lowest amount of copies in the genome.

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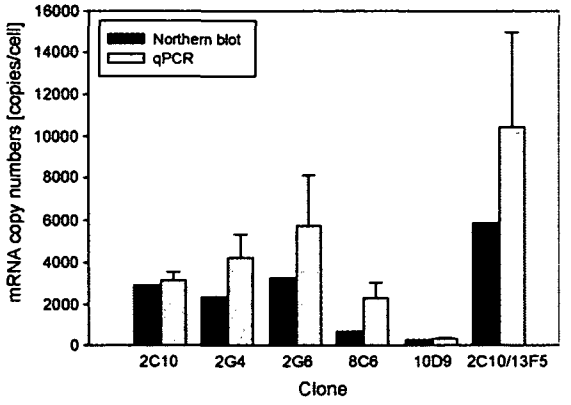

Fig. 6. mRNA-levels determined by qPCR are higher than in Northern blot. Clone 10D9 exhibited only a few hundred copies while clone 2C10/13F5 was in the range of 10,000 copies of EpoFc transcript per cell.

qPCR, the low sensitivity of blot methods is evident, that is also reported by Malinen et al., 2003; Bustin, 2000 and Dean et al., 2002. Therefore we prefer qPCR for analysis of GCN of clones that have not undergone several steps of MTX amplification. Standard deviations in our Southern blot were up to four times higher than in quantitative PCR that can be explained by the semi-quantitative detection method of the Lumi-Imager. Concerning qPCR, Bubner et al. (2004) claims that two-fold differences in GCN are the quantification limits, which is comparable to max. 37% of standard deviation obtained in our experiments. The problems of quantitative PCR arise predominantly from the back transformation of the linear model of threshold levels to real copy numbers, since quantitative PCR follows exponential growth kinetics.

In case of mRNA quantification we compared the Northern blot technique to the Taqman quantitative PCR (see Fig. 6). The qPCR results were up to three times higher compared to the data obtained from two independent Northern blot experiments. However, both methods can be set in direct correlation ($R^2 = 0.93$; data not shown). In case of Northern blot analyses the quantification is done by comparison of RNA samples with plasmid standards which might be responsible for methodological errors. Therefore we will refer to the mRNA copy number determined by qPCR for further discussion.

3.6. Influence of GCN and mRNA copy number on q_p

Table 2 summarises the genomic and transcriptomic parameters determined by Taqman quantitative PCR as well as specific productivities. Clones 2C10 and 2G6 showed similar specific productivities and gene copy numbers but different specific mRNA levels. 2G6 has an approximately two-fold higher amount of EpoFc transcript, but only a 15% higher EpoFc secretion rate. We assume that in case of such a complicated product like EpoFc, translation and secretion of correctly folded protein is a further bottleneck in recombinant protein expression. When looking at clones 8C6 and 2G4, same restriction patterns as well as similar relations between GCN and mRNA level, as well as mRNA copy number and q_p is evident, but show in contrast to 2C10 and 2G6 a significantly lower product secretion. Therefore we suggest a problem during translation resulting in accumulation of EpoFc mRNA in the cell. Concerning

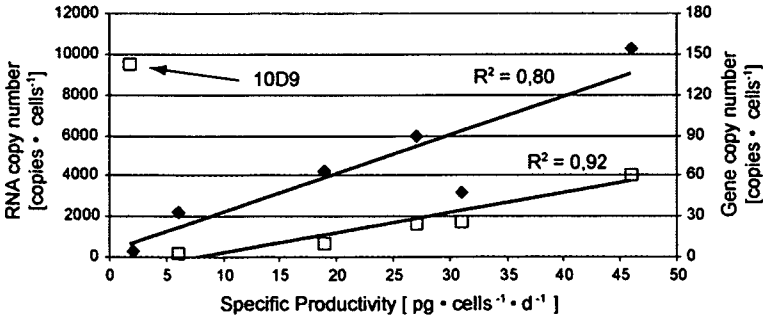

Fig. 7. Correlation of genetic parameters (GCN (□) and mRNA copy number (♦)) with specific productivities. By omitting clone 10D9, correlation of GCN with q_p resulted in a coefficient of 0.92. A similar correlation was obtained for the mRNA copy number with specific productivity (0.80).

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clone 10D9, we observed the highest gene copy number but lowest specific productivity that is also reflected in the lowest mRNA level. This effect might be due to incomplete expression cassettes or integration of the exogene into inactive loci.

In case of the best producing clone, 2C10/13F5, we found a 70% higher q_p compared to the mother clone; however, the GCN and the amount of EpoFc transcript were disproportionately high. This is a good example for successful gene amplification by maintaining the same integration locus leading to a higher gene copy number.

To summarise, Fig. 7 shows a good correlation of specific productivities with specific mRNA-levels ($R^2 = 0.80$). In contrast, a correlation of GCN versus q_p could not be generally observed. Nevertheless, after omitting clone 10D9 a correlation of 0.92 was achieved. Therefore an influence of transcript copy number on specific productivity was clearly evident and on the other hand a high GCN not necessarily resulted in productivity.

4. Conclusion

In this paper we have shown that our EpoFc expressing primary transfectants reached respectable productivities (19–31 pg cell^{-1} d^{-1}) with transgene copy numbers from 10 to 26 copies/cell, in contrast to other studies where 100–1000 copies of insert were detected after multiple rounds of amplification (Yoshikawa et al., 2000; Davies et al., 2001; Weidle et al., 1988; Dixkens et al., 1998).

Our clones did not undergo several steps of amplification and the low MTX pressure (0.1 μM MTX was used for gene amplification) favours clonal stability over a long period of time. Stability in specific productivity could be demonstrated in spinner cultivation over several months after revitalisation from a research cell bank, and furthermore q_p remained constant over five repeated batches in bioreactor cultivation (Trummer et al., 2006).

Based on our data gained from a typical example of recombinant cell line development, discussion of mRNA copy number together with GCN and q_p is inevitable in order to get information about bottlenecks during transcription, translation and secretion. Screening for the most balanced clone during selection for subcloning, amplification, protein-free adaptation and fermentation might reduce the risk of productivity decreases and instabilities during the scale-up process.

Nevertheless, this is a very complicated field and very limited data have been published so far in the wider Biotech-community. Further comparisons of clones have to be done to implement analyses of genetic parameters into the selection and screening process of recombinant cell lines.

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ORIGINAL PAPER

Identification of transgene integration loci of different highly expressing recombinant CHO cell lines by FISH

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Abstract Recombinant CHO cell lines have integrated the expression vectors in various parts of the genome leading to different levels of gene amplification, productivity and stability of protein expression. Identification of insertion sites where gene amplification is possible and the transcription rate is high may lead to systems of site-directed integration and will significantly reduce the process for the generation of stably and highly expressing recombinant cell lines. We have investigated a broad range of recombinant cell lines by FISH analysis and Giemsa–Trypsin banding and analysed their integration loci with regard to the extent of methotrexate pressure, transfection methods, promoters and protein productivities. To summarise, we found that the majority of our high producing recombinant CHO cell lines had integrated the expression construct on a larger chromosome of the genome. Furthermore, except from two cell lines, the exogene was integrated at a single site. The dhfr selection marker was co-localised to the target gene.

Keywords FISH · Giemsa–Trypsin banding · Insertion locus · Integration site · Recombinant CHO

Abbreviations
DIG digoxigenin
GCN gene copy number
HGP highly glycosylated protein
MCB master cell bank

Introduction

Generation of recombinant mammalian cell lines for production of protein pharmaceuticals is driven by various factors like the gene transfer vector, the host cell line and the transfection method. An efficient combination of selection, screening and gene amplification leads to stably and highly producing clones ready to be propagated in scalable reactors. Various studies (Davies et al. 2001; Kaufman et al. 1983; Kim et al. 1999; Pallavicini et al. 1990; Yoshikawa et al. 2000a, b; Derouazi et al. 2006) have investigated the influence of the integration locus on stability

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and productivity of the clones in order to obtain an additional criterion for clone selection since the majority of random integration sites leads to low level expression (Barnett et al. 1995). Furthermore, information of insertion loci which allow gene amplification and support high productivity and stability—in addition with systems for site-directed integration (the Flp/FRT system from the yeast *Saccharomyces cerevisiae* (O'Gorman et al. 1991) and the CRE/lox system (Hoess et al. 1982) from the bacteriophage P1)—might significantly reduce the selection and screening process. Therefore, some authors recommend additional selection parameters on the chromosomal level.

Different factors that might be related to the integration site have been suggested to influence the expression level: Wilson et al. (1990) assumes a "position effect", which is either caused by rearrangement or incorporation of recombinant genes into regions with nearby endogenous promoters and enhancers which support gene expression. Furthermore, various DNA elements (Barnes et al. 2002) can modulate the position effect: insulators, MAR elements, locus control regions and ubiquitous chromatin opening elements may be important in controlling expression levels and stability of recombinant protein production. The integration of the target gene within heterochromatic regions of DNA, which arise due to the dense packaging of DNA and associated proteins, are generally considered to be transcriptionally inactive. Palin et al. (1998) demonstrated that stable transfectants had preferentially integrated the ingoing plasmid DNA into fragile-site containing chromosomal bands. These sites are reproducibly expressed and chemically induced decondensations on mitotic chromosomes with high recombinogenic activity. Analysis of the flanking regions revealed a highly AT-rich region and DNA sequence motifs and structurally distinct regions associated with replicative origins immediately adjacent to the integration site. These regions are frequently associated with sister chromatide exchange events and can be activated in vivo and trigger specific chromosomal breaks leading to intrachromosomal mammalian gene amplification (Kuo et al. 1994).

The progress of gene amplification is assumed to be initiated either by chromosome breakage within expanded chromosomal regions, also called homogenously stained regions (HSRs) that fail to exhibit trypsin-giemsa bands (Kaufman et al. 1983; Nunberg et al. 1978), or in extrachromosomal elements, also known as acentric chromatin bodies or double minute chromosomes (DMs; Brown et al. 1981; Kaufman et al. 1979), furthermore in abnormally banded regions (ABRs) or in dicentric chromosomes. Whereas association of amplified DNA with HSRs is often observed in highly and stably producing cell lines (Kaufman et al. 1985), amplified genes localised to DMs are often lost, especially in the absence of selective pressure (Kaufman et al. 1983; Wahl et al. 1982).

Concerning the number of insertion sites, Davies et al. (2001) suggest, that cell lines with only one integration locus are important in the development of highly productive clones, although subsequent gene amplification by increasing the selection pressure either often leads to translocations with multiple hybridisation signals on either the same or different chromosomes (Davies et al. 2001; Pallavicini et al. 1990), or to the arising of new marker chromosomes or to the enlargement of chromosomes. Furthermore, signals on small derivative chromosomes or fragments are reported (Pallavicini et al. 1990), and small chromosomes might disappear (Kaufman et al. 1985).

Yoshikawa et al. (2000a, b) investigated the location of the insertion site on the chromosome and analysed a recombinant cell line adapted to different concentrations of Methotrexate (MTX) and distinguished three different types of recombinant cell lines regarding the insertion site of the transgene: telomeric type cells had integrated the exogenous target together with the selection marker into the telomeric region, while the two other groups showed integration to other chromosomal regions or did not show any signal in FISH analysis. Whereas the telomeric type clones exhibited high productivities, growth rates and stable integration, lower productivity and unstable heterogeneous cell pools were put into relation with insertion into other regions.

In our institute, several recombinant CHO cell lines with stable productivities have been obtained after a different number of subcloning

and amplification steps. In contrast to other studies, where only analysis of subclones differing in the extent of MTX amplification was performed, we investigated the integration patterns of a broad range of cell lines by FISH analysis. In order to distinguish between the different chromosomes, the method of centromer localisation was applied and therefore the following terms will be used: metacentric (chromosomes with centromers in the middle resulting in two arms with equal lengths), submetacentric (centromers between the middle and the end of the chromosome), acrocentric (centromer close to the end of the chromosome) and telocentric (centromer at the end resulting in only one arm). By Giemsa–Trypsin banding the karyotype of each cell line was analysed and allows conclusions about the amplification mechanism and karyotypic stability. Based on these results we will discuss the obtained integration sites in connection with specific productivities and the extent of MTX amplification.

Material and methods

Recombinant CHO cell lines

Table 1 gives an overview of the different stably producing recombinant CHO cell lines that were analysed. Dihydrofolate reductase-deficient Chinese Hamster Ovary Cells DUK-, ATCC CRL-9096 (Urlaub et al. 1980) were either transfected under serum-dependent conditions and later adapted to protein-free cultivation or were already transfected under protein-free conditions. Increase in MTX pressure was performed gradually in 2- or 4-fold steps. Cultivation of the cell lines by applying the MTX pressure indicated in Table 1 was performed after revitalisation from a frozen cell bank. The number of passages between revitalisation and analyses is also shown in Table 1. In general, EpoFc cell lines were cultivated over five passages before genetic analyses were performed, whereas the other recombinant production cell lines were cultivated about 20–30 passages after revitalisation from a MCB before the integration sites were investigated.

For determination of specific productivity, cell concentration was determined in a Multisizer™ 3 Coulter Counter® (Beckman Coulter) and the secreted product concentration was quantified using an ELISA.

FISH

The probes for FISH-experiments were generated by overnight DIG-Klenow Labelling of 3 µg of the transfected plasmids using DIG-High Prime (Roche, Cat.Nr. 1585606). The labelled plasmids were co-precipitated with 150 µg Hering Sperm DNA (Roche, Cat.Nr. 223646) and 150 µg Yeast t-RNA (Roche, Cat.Nr. 109495) in 0.3 M Sodium-Acetate (pH 5.2) after addition of 2.5 volumes of 96% ethanol. Finally, the precipitated probes were dissolved in 300 µl of Probe Stock Solution (50% Formamide, 2× SSC, 10% Dextransulphate, 50 mM Sodiumphosphate pH 7.0).

For preparation of metaphase spreads suspension cells were incubated for 6 h in Demecolcine (Sigma, Cat.Nr. D-6165; 0.2 µg per ml cell suspension). Cells were harvested by centrifugation at 190 *g*, resuspended in 5 ml of a 75 mM KCl solution and incubated at 37°C for 20 min. After centrifugation, cells were washed three times in 5 ml of fixation solution (MetOH/Acetic Acid, 3:1) and finally resuspended to a final concentration of 5×10^6 cells ml^{-1} in fixation solution.

Slides were cleaned in 6 N HCl and 96% ethanol and stored at 4°C in water. The fixed cells were dropped on wet and cold slides over a 65°C water bath, where they were left for 1 min before they were dried at room temperature.

To reduce background staining, the slides were incubated in 0.02% Pepsin in 10 mM HCl for 10 min at 37°C, then washed 2 times for 5 min in PBS, once for 5 min in PBS/50 mM $MgCl_2$ and finally for 10 min in PBS/50 mM $MgCl_2$/1% Formaldehyde. After two brief washings in PBS, the slides were dehydrated in a graded series of ethanol (70%/90%/96%).

The probes were diluted 1:5 in Probe Stock solution to a final concentration of 2 ng μl^{-1}. The slides were incubated overnight at 37°C with 25 µl of this solution under a cover slip fixed with Fixogum (Marabu) after denaturation at 90°C for 5 min.

Table 1 Overview of the investigated cell lines concerning the recombinant product, the transfection method, the plasmids and the extent of MTX amplification

Name	Product	Transfection method	Plasmids	MTX-pressure (μM)	Number of subcloning steps	Passages after revitalisation	Specific productivity (pg cell^{-1} day^{-1})
EpoFc 2C10				0.1	0	5	27
EpoFc 2C10/13F5				0.4	1	5	46
EpoFc 2G4		Lipofection	pCMV_EpoFc	0.1	0	5	19
EpoFc 2G6	Fusion-protein			0.1	0	5	31
EpoFc 10D9				0.1	0	5	2
EpoFc HI5		Protein-free	pCMV_EpoFc	0.1	0	5	11
EpoFc HI5/7D3		Nucleofection		0.2	1	5	35
IgM Hb617	Antibody	Lipofection	pIRES CMV dhfr HC pIRES CMV JC LC	0.1	2	5	25
IgG 2F5	Anti-HIV IgG	Calcium Phosphate Coprecipitation	pRC/RSV LC pRC/RSV HC	1.0	3	37	10
IgG 2F5 sf	Anti-HIV IgG	Protein-free Nucleofection	pRC/RSV LC pRC/RSV HC	1.5	2	31	4
IgG 2G12	Anti-HIV IgG	Lipofection	pRC/RSV LC pRC/RSV HC	1.0	4	21	10
IgG 4E10	Anti-HIV IgG	Lipofection	pCI neo CMV LC pCI neo CMV HC	0.4	2	18	15
Fab 3H6	Fab Fragments	Protein-free Nucleofection	pIRES CMV HC LC pIRES CMV LC dhfr	1.5	2	24	10
HGP	Highly glycosylated human protein	Lipofection	pSV40 HGP	0.4	2	34	22

Furthermore, the number of subcloning steps as well as of the passages between revitalisation from a cell bank and analysis is shown. In the last column the specific productivity of each cell line is present. Abbreviations: HC, heavy chain; LC, light chain; JC, joining chain; CMV, cytomegalovirus promoter; RSV, Rous sarcoma virus promoter; SV40, Simian virus 40 promoter. Except for the cell lines transfected with pIRES that already contained dhfr, an additional plasmid encoding mouse dhfr was cotransfected

Slides were then washed three times for 5 min in 2× SSC pH 7.0%/50% Formamide at 45°C, then 5 times for 2 min 2× SSC pH 7.0, then once for 5 min in TNT-Washing Buffer (100 mM Tris Cl/150 mM NaCl pH 7.5, 0.05 % (v/v) Tween 20 (Roche, Cat.Nr. 1332465)). Afterwards slides were incubated for 30 min at 37°C in TNB-Blocking Buffer (0.5% (w/v) Blocking Reagent (Roche, Cat.Nr. 1096176) in 100 mM Tris Cl/150 mM NaCl pH 7.5). Chromosomes were then incubated for 30 min at 37°C in Antibody Solution 1 (Mouse-Anti-DIG-Antibody (Roche, Cat.Nr. 1333062) 1:250 in TNB-Blocking Buffer) and after three washes in TNT-Washing Buffer for 30 min at 37°C in Antibody Solution 2 (Goat-Anti-Mouse-FITC-Antibody (Sigma, Cat.Nr. F-2012) 1:64 in TNB-Blocking Buffer). Slides were again washed three times and incubated for 30 min at 37°C in Antibody Solution 3 (Rabbit-Anti-Goat-FITC-Antibody (Sigma, Cat.Nr. F-7367) 1:64 in TNB-Blocking Buffer). After three final washings, chromosomes were dehydrated in a graded series of ethanol. Air dried chromosomes were counterstained in Propidium Iodide (0.5 μg ml^{-1}, Sigma, Cat.Nr. P-4170), embedded in Vectashield (Vector, Cat.Nr. H-1000) and examined in a confocal microscope (Leica TCS SP2). As a negative control, the host cell line was hybridised with the probes. At least 20 FISH preparations were analysed.

Giemsa–Trypsin banding

After preparation of metaphase spreads on slides as described above, chromosomes were stored at room temperature for one week in order to enable aging. The following solutions were used for banding: GKN buffer (1.0 g l^{-1} Glucose, 0.4 g l^{-1} KCl, 8.0 g l^{-1} NaCl, 0.35 g l^{-1} $NaHCO_3$), Versen buffer (8.0 g·l^{-1} NaCl, 0.2 g l^{-1} KH_2PO_4, 0.2 g l^{-1} KCl, 1.44 g l^{-1} $Na_2HPO_4 \cdot 2H_2O$), Trypsin Stock Solution (2.5% Trypsin (1:250, Gibco, Cat.Nr. 27250-018) in 0.9% NaCl).

Slides were then first incubated at 30°C for 45 s in trypsin solution (add 132 ml GKN buffer, 24 ml 0.9% NaCl and 4 ml Trypsin Stock Solution to 165 ml Versen buffer), then washed briefly three times in GKN supplemented with 7.5% FCS and four times in GKN buffer before staining in 8% Giemsa Solution (Merck, Cat.Nr. 109204) for 5 min. Slides were rinsed with water and finally analysed under the microscope.

Investigation of genetic parameters

Isolation of genomic DNA was performed by using the QIAamp® DNA Blood Mini Kit (Qiagen) according to the manufacturer's instructions. For determination of the gene copy number (GCN), 6 ng of genomic DNA were analysed by Taqman quantitative PCR in the Rotor-Gene 2000 (Corbett Research) using the iQ Supermix (Biorad). The plasmid standard pCMV_EpoFc (5455 bp, stock solutions of 1×10^8 copies μl^{-1} stored at –20°C) was used in a range from 3×10^6 to 3×10^3 copies in a 1:10 dilution series analysed in duplicates.

For determination of mRNA copy number by quantitative PCR, RNA was isolated from 2.5×10^6 cells using the RNeasy Mini Kit (Qiagen). After DNase treatment and reverse transcription, cDNA was purified by using the QIAquick PCR Purification Kit (Qiagen). Concentration was quantified spectrophotometrically at 260 nm. cDNA was adjusted to a concentration of 20 ng copies μl^{-1} and stored at –20°C. About 6 ng of cDNA were analysed as described for genomic DNA. Calculation of the transcript copy was based on the quotient $q_{cDNA/RNA}$ obtained from the amounts of reversely transcribed RNA and resulting cDNA. Finally, the initial cell number analysed in quantitative PCR was recalculated by the average amount of 12.2 pg of total RNA per cell.

For Southern Blot analysis *Bam*HI digested genomic DNA of each clone was separated on a 1% agarose gel, denatured and transferred to a positively charged nylon membrane according to the Roche Manual. After prehybridisation (7% SDS, 50% Formamide, 5 × SSC, 2% Blocking Reagent, 50 mM Sodium-phosphate pH 7.0, 0.1% *N*-Lauroylsarcosine), the membrane was incubated in hybridisation buffer containing 10 ng ml^{-1} of the denatured probe (a DIG-labelled Fc specific amplicon) for at least 12 h at 42°C. Washing and detection was performed as described by Roche (DIG Application Manual for Filter Hybridization) using Anti-Digoxigenin-AP Fab fragments (Roche) and

CPD-Star® (Tropix). Visualisation was done on the LumiImager™ (Roche).

Results and discussion

Analysis of the EpoFc clone family

The FISH signals are shown in Fig. 1. For detailed analysis of the position on the chromosomes, phase contrast microscopic images (not shown) were used. For clones EpoFc 2C10, 2G6 and the subclone 2C10/13F5 (Fig. 1a–c) a single hybridisation signal is evident on the large arm of an acrocentric chromosome near the telomeric region. Analysis of clone 2G4 (Fig. 1d) revealed also a single signal but on the large arm of submetacentric chromosome. In case of the protein-free transfected clone HI5 (Fig. 1e) and its subclone HI5/7D3 (Fig. 1f) two populations were observed, one of them (about 90%) bearing a single integration signal again on a acrocentric chromosome, while the other population (about 10%) revealed an additional signal on a metacentric chromosome. Clone 10D9 (Fig. 1g) was the only clone that exhibited a signal on the short arm of an acrocentric chromosome. Analysis of the integration site of the cotransfected dhfr gene (as expression vectors with different backbones have been used for transfection, no unspecific signal could be obtained) revealed co-localisation to the integration site of the target gene (pictures not shown), which is reported in several studies (Pallavicini et al. 1990; Chen et al. 1998; Strutzenberger et al. 1999; Kim et al. 1999).

Analysis of the chromosome number (at least 20 metaphase preparations were counted) of the EpoFc clone family revealed an average diploid chromosome number of 20 chromosomes for clones 2C10, 2G4 and 2G6. Clone HI5 and the two subclones, 2C10/13F5 and HI5/7D3 had only 19 chromosomes, whereas for clone 10D9 21 chromosomes were evident. For comparison with the host cell line—that contained 20 chromosomes, Giemsa–Trypsin banded chromosomes were sorted by their size, then banding patterns were used to arrange chromosomes (Fig. 2). No efforts were made in order to specifically identify

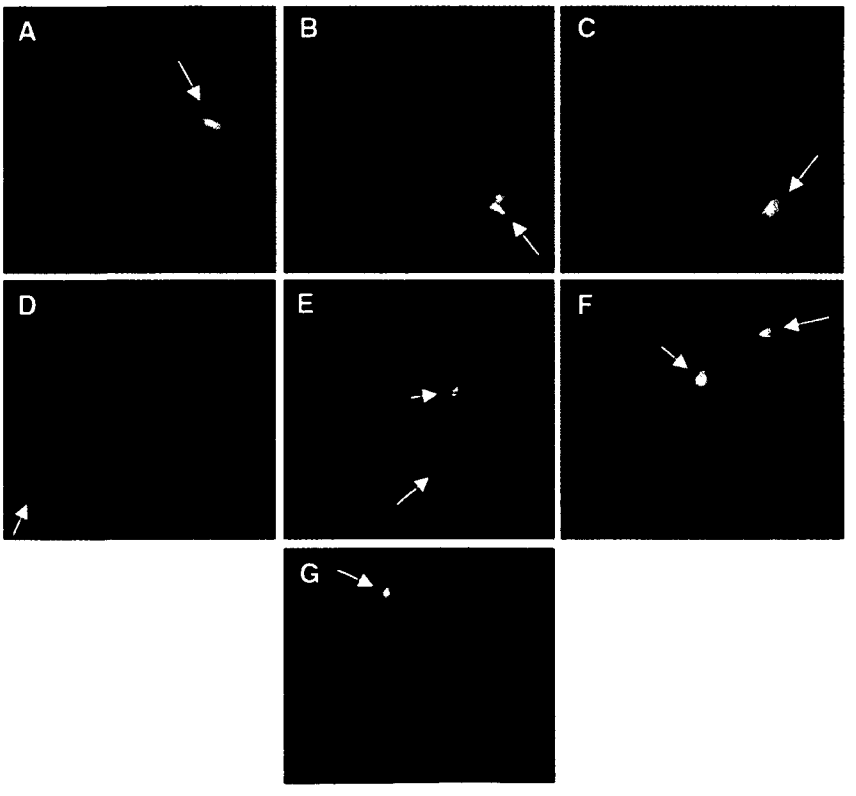

Fig. 1 Hybridisation patterns of the EpoFc clones: 2C10 (**a**), 2G6 (**b**), 2C10/13F5 (**c**), 2G4 (**d**), HI5 (**e**), HI5/7D3 (**f**) and 10D9 (**g**). Integration sites are marked with a white arrow

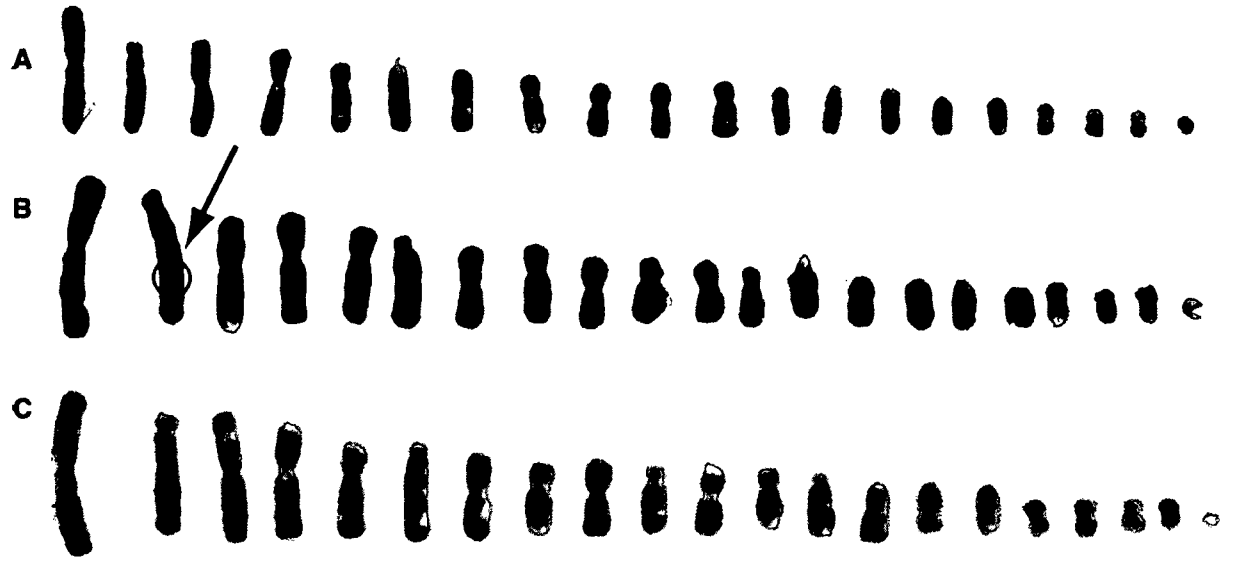

Fig. 2 Giemsa–Trypsin banding patterns of selected EpoFc clones compared to the host cell line (**a**): EpoFc 2C10 (**b**) and EpoFc 10D9 (**c**). The site of integration in EpoFc 2C10 is marked with an arrow, whereas it could not be identified in EpoFc 10D9 since the signal on the short arm of an acrocentric chromosome could not be related to a certain chromosome. FISH-analysis on banded chromosomes might give further information about the integration locus

the banded chromosomes with regard to the standard CHO karyotype, as the karyotype of the CHO DUK- host cell line differs significantly from that of other CHO cells, probably due to the extensive UV ray and ethyl methane sulphonate mutagenesis used to derive the dfhr deficient line (Varshavsky 1981).

Since similar banding patterns were found in clones EpoFc 2C10 (Fig. 2b) and 10D9 (Fig. 2c) compared to the host cell line (Fig. 2a), we assume that no translocations have occurred. As the hybridisation signal in clone 2C10 was observed on the largest acrocentric chromosome (localisation was performed by transmission light microscopy of the hybridised slides), and no other chromosome with similar size could be detected, the integration site is on chromosome 2 in a dark, in comparison to the host, slightly expanded, homogenously stained region. In clone EpoFc 10D9, localisation of the signal on the short arm of an acrocentric chromosome is not possible. Concerning clone EpoFc 2G6 and the subclone EpoFc 2C10/13F5 the signal was also found on chromosome 2 (data not shown).

EpoFc expressing clones were further analysed for gene copy number and specific mRNA content of the target gene by Taqman quantitative PCR and resulted in the following copy numbers (see Table 2).

Table 2 Specific productivities, gene and mRNA copy numbers of the investigated EpoFc clones

	q_p (pg 10^{-6} cells day^{-1})	GCN (copies·$cell^{-1}$)	mRNA copy number (copies·$cell^{-1}$)
EpoFc 2C10	27	26	3143
EpoFc 2G6	31	25	5920
EpoFc 2C10/ 13F5	46	61	10263
EpoFc 2G4	19	10	4220
EpoFc HI5/7D3	35	12	2402
EpoFc 10D9	2	140	334

HI5 was not analysed for genetic parameters and is therefore not listed in the table. EpoFc HI5/7D3 is the only clone already transfected under protein-free conditions that was investigated, the other five clones were analysed after protein-free adaptation. Clone EpoFc HI5/7D3 evaluated comparable specific productivities as clone EpoFc 2G6, although the GCN and the amount of transcript were significantly lower. The reason for this phenomenon might be either the different integration site, or a more efficient mRNA/protein-processing machinery of clones that were already transfected under protein-free conditions

Concerning the gene copy numbers, values in the range of 10–26 copies per cell were obtained, only in case of EpoFc 10D9, 140 copies per cell were detected, and for the subclone EpoFc 2C10/13F5 after MTX amplification the gene copy number was increased to 60 copies per cell.

When looking at FISH analyses, signal intensities do not correlate with gene copy numbers: Clone EpoFc 2G6 (Fig. 1b) exhibited a rather broad signal in comparison to clone EpoFc 10D9 (Fig. 1g), although determination of the gene copy number showed a sixfold higher amount of EpoFc genes for clone EpoFc 10D9. These differences can be explained by the more difficult accessibility in FISH analysis due to the condensed packaging of chromatin in metaphase cells and in the signal bleaching during laser microscopy. Therefore, FISH hybridisation can not be adopted as a (semi-)quantitative method for determination of the GCN but rather gives some hints about rearrangements and clonal stability and homogeneity of the cell population.

However, as shown in Table 2, mRNA copy numbers do not correlate in general with the GCNs in various clones. This observation strongly supports that mRNA transcription is predominantly driven by the integration site and the surrounding locus—a phenomenon that is also described as "position effect" (Wilson et al. 1990).

First information concerning the insertion locus was gained by Southern Blot analysis. The resulting restriction patterns are demonstrated in Fig. 3. Due to the same sizes of the restriction fragments obtained for clones EpoFc 2C10 and 2G6, we suggested same integration sites, which could be confirmed in FISH analyses and Giemsa–Trypsin banding, where a signal was detected on the second largest chromosome for both clones. The distal end of the large arm of chromosome 2 was also reported as the major integration site by Pallavicini et al. (1990), and Derouazi et al. (2006) also detected larger chromosomes as the major integration sites in recombinant CHO DG44 cell lines.

The high gene copy number found in qPCR for clone EpoFc 10D9 was confirmed by Southern Blot analysis. EpoFc 10D9 gave higher signals compared to EpoFc 2G6 but the restriction pattern differed significantly from all other investigated clones (see Fig. 3). Therefore, the FISH signal on the small arm of an acrocentric chromosome of clone EpoFc 10D9 correlates with the different Southern Blot results.

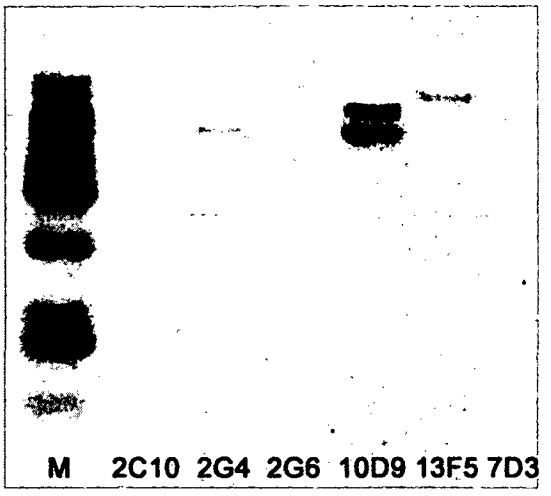

Fig. 3 Southern blot after digestion of 1.5 µg of genomic DNA for EpoFc clones (4 µg for clone EpoFc HI5/7D3): M: λ DNA/*Eco*RI + *Hin*dIII marker; further lanes show the genomic DNA of the clones. Intensities correlate well with gene copy numbers determined by quantitative PCR, but not with signal intensities detected in FISH analyses

Analysis of further recombinant cell lines

Analysis of metaphase spreads (at least 20 preparations were counted) revealed 16 chromosomes for clone IgG 2F5, 18 chromosomes for the protein-free transfected IgG 2F5sf and 19 chromosomes for clones IgG 2G12 and 4E10, Fab 3H6 and the cell line expressing a highly glycosylated human protein, HGP. IgM Hb617 was tetraploid in a certain extent, with a diploid chromosome number of 18. Clone IgG 2G12 was completely aneuploid with a chromosome number around 30.

In general we do not recommend classifying the CHO karyotype into diploid and tetraploid, since the hamster (*Cricetulus griseus*) chromosome should have 22 chromosomes (Ray et al. 1976), and the parental CHO cell line (CHO-K1) has only 21 chromosomes (Kao et al. 1968). The cell line we used as host cell line had 20 chromosomes.

Hybridisation signals of antibody and glycoprotein producing cell lines are shown in Fig. 4. Except for IgG 2G12, a single hybridisation signal was detected: while clone IgG 2F5 showed an extensive fluorescent region on the distal end of the longer arm of the submetacentric chromosome 1 (Fig. 4a), hybridisation of the protein-free transfected IgG 2F5sf revealed the signal on a small metacentric chromosome on the telomeric end (Fig. 4b).

Clone IgG 2G12 is aneuploid and shows up to five integration sites (Fig. 4c)—interestingly it is the only clone where integration into centromeric parts of small chromosomes occurred. As only the number of small chromosomes increased in comparison to the host cell line, we suggest that integration of the target into centromeric regions can lead to chromosomal instabilities resulting in chromosomal duplication. In addition, Yoshikawa et al. (2000a) suggested, that at non-telomeric integration sites gene amplification leads to replication and sister chromatide exchange whereas in telomere type clones amplification is achieved by the fusion of two telomere regions.

In contrast to clone IgG 2F5, clones IgG 4E10 (Fig. 4d), IgG Hb617 (Fig. 4e) and Fab 3H6 (Fig. 4f) integrated the plasmid into the large arm of the largest acrocentric chromosome. Finally, the last cell line, producing the glycoprotein HGP, exhibited the signal again on the larger arm of an acrocentric chromosome (Fig. 4g).

For comparison with the initial host cell line karyotype, Giemsa–Trypsin banding was performed. Figure 5 shows the banding patterns of two cell lines (IgG 2F5 and HGP). Whereas in the HGP cell line (Fig. 5c) chromosomes can be easily identified, in IgG 2F5 producing cells (Fig. 5b) the banding pattern differs from the host cell line. Furthermore, IgG 2F5 had only 16 chromosomes in contrast to 20 chromosomes for the host cell line and 19 chromosomes for the HGP cell line. This might be due to the adaptation to different MTX concentrations: 0.4 μM MTX in case of HGP and 1.0 μM MTX in case of IgG 2F5. Therefore, we suggest, that the increase

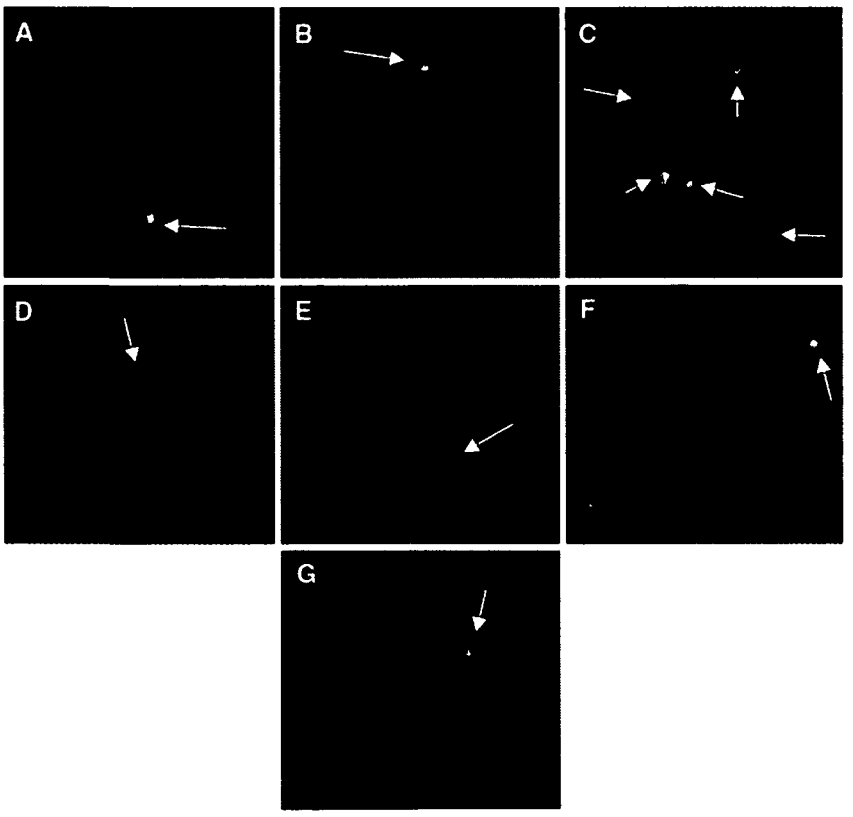

Fig. 4 Hybridisation patterns of the following clones: IgG 2F5 (**a**), IgG 2F5 sf (**b**), IgG 2G12 (**c**), IgG 4E10 (**d**), IgM Hb617 (**e**), Fab 3H6 (**f**), HGP (**g**). Integration sites are marked with a white arrow

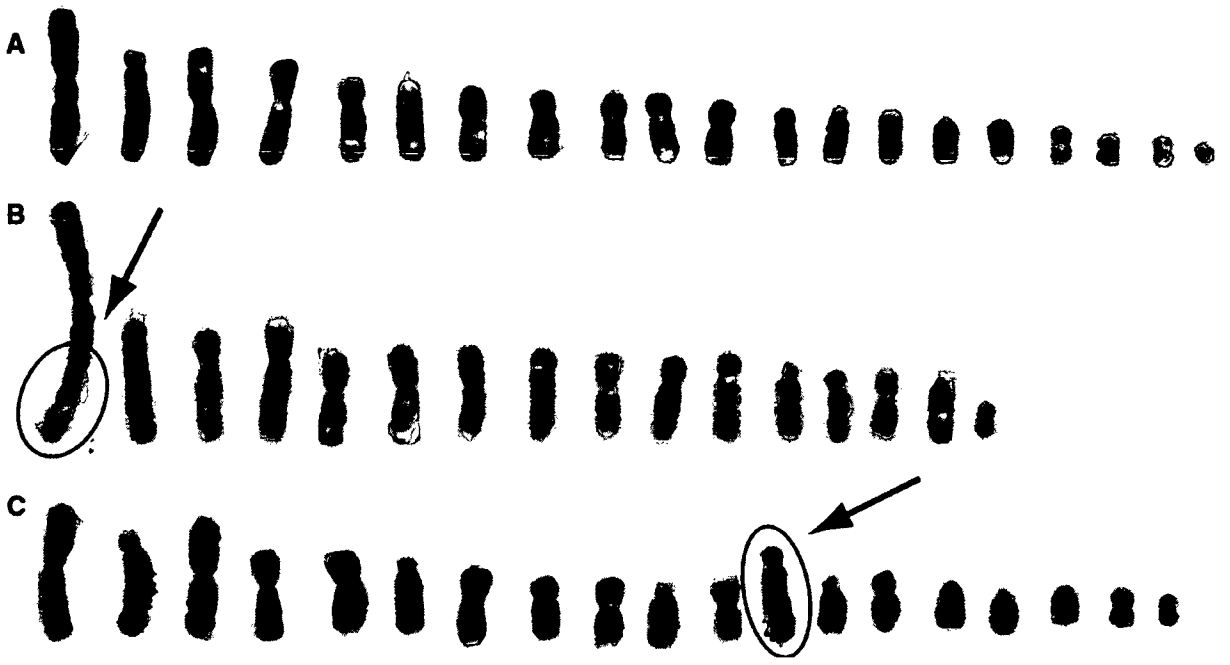

Fig. 5 Giemsa–Trypsin banding patterns of the clones compared to the host cell line (**a**): IgG 2F5 (**b**) and HGP (**c**). The sites of integration are marked with an arrow

in selection pressure leads either to the loss of small chromosomes, or—as observed for clone IgG 2G12, where the same amplification steps were applied—to karyotypic instabilities leading to aneuploidy. Nevertheless, in case of these clones, no effect on stability of protein production was observed. Derouazi et al. (2006) also showed that chromosomal aberrations frequently occurred during generation of recombinant CHO cell lines regardless of the gene transfer method and did not correlate with the stability of recombinant protein expression.

In addition, when looking on the site of integration in the giemsa-banded chromosomes of clone IgG 2F5 (see Fig. 5b), a homogeneously staining region is evident. Looking at clone HGP (Fig. 5c), a new phenomenon was observed: in comparison to the host cell line an acrocentric chromosome similar in size to chromosome 2 was found, although the Giemsa–Trypsin banding pattern was similar to a smaller sized chromosome. In FISH analysis the signal was detected on one of these two chromosomes, due to the fact that gene amplification might lead to translocation or expanded chromosomal regions, we assume that the target is localised to the new marker chromosome.

Conclusion

Cultivation of EpoFc cell lines was performed over a shorter period of time compared to the antibody and HGP producing cell lines. Nevertheless, as these cell lines were also cultivated in the presence of MTX and productivity remained stable during that time, comparison with EpoFc cell lines is justified.

To summarise, after analysis of a wide range of recombinant cell lines the localisation of the integrated genes can be divided into several groups: Clone IgG 2F5 integrated the exogenous target into chromosome 1, whereas for clone EpoFc 2C10 and its subclone, 2C10/13F5, EpoFc 2G6, Hb617, 4E10 and 3H6 Fab the insertion locus was found on chromosome 2. Clone EpoFc HI5 and its subclone (HI5/7D3) exhibited the hybridisation signal on either one or two middle-sized chromosomes, whereas clone EpoFc 10D9 had the integration locus

on the smaller arm of a middle-sized acrocentric chromosome. The signal of clone HGP was detected on a new marker chromosome, in clones EpoFc 2G4 and IgG 2F5 sf integration into a smaller chromosome occurred. Clone IgG 2G12 was the only clone where insertion loci were co-localised with the centromeric region of five chromosomes.

Looking at the cell lines with insertion into chromosome 2, two different methods of transfection (lipofection and protein-free nucleofection) with genes under the control of two different promoters (CMV and RSV) have been applied. Therefore, we suggest that no effect of the transfection method and promoters on the integration site are evident.

As integration not only into the telomeric sites of the chromosomes occurred, the theory of Yoshikawa et al. (2000a, b) who assumes that only integration into the telomeric region leads to high productivity and stability cannot be confirmed—stable producing cell lines with integration of the exogenous target into other areas of the chromosomes were also reported by Derouazi et al. (2006).

Despite the difficulty of comparing specific productivities of cell lines expressing different products, the clones with the insertion locus on chromosome 2 exhibited (except from clone Fab 3H6) high productivities with a rather low extent of MTX amplification. Therefore, we assume that integration into this region enhances transcription efficiency.

Sequence analysis of the surrounding regions might give further insight of potential transcription control elements.

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