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

Christine Lattenmayer · Martina Loeschel · Willibald Steinfellner ·
Evelyn Trummer · Dethardt Mueller · Kornelia Schriebl ·
Karola Vorauer-Uhl · Hermann Katinger · Renate Kunert

Received: 9 August 2006 / Accepted: 4 October 2006 / Published online: 15 November 2006
© Springer Science+Business Media B.V. 2006

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

C. Lattenmayer (✉) · E. Trummer · K. Schriebl
Austrian Center of Biopharmaceutical Technology,
Muthgasse 18, 1190 Vienna, Austria
e-mail: christine.lattenmayer@malteser.at

W. Steinfellner · D. Mueller · K. Vorauer-Uhl ·
H. Katinger · R. Kunert
Department of Biotechnology, Institute of Applied
Microbiology, Muthgasse 18, 1190 Vienna, Austria

M. Loeschel · H. Katinger
Polymun Scientific, Immunbiologische Forschung,
Nussdorfer Laende 11, 1190 Vienna, Austria

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% Dextranulphate, 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 MgCl₂ and finally for 10 min in PBS/50 mM MgCl₂/1% 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 ($\text{pg cell}^{-1} \text{day}^{-1}$)
EpoFc 2C10				0.1	0	5	27
EpoFc 2C10/13F5				0.4	1	5	46
EpoFc 2G4			pCMV_EpoFc	0.1	0	5	19
EpoFc 2G6	Fusion-protein	Lipofection		0.1	0	5	31
EpoFc 10D9				0.1	0	5	2
EpoFc H15		Protein-free	pCMV_EpoFc	0.1	0	5	11
EpoFc H15/7D3		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	pRC/RSV LC pRC/RSV HC	1.0	3	37	10
IgG 2F5 sf	Anti-HIV IgG	Coprecipitation					
		Protein-free	pRC/RSV LC	1.5	2	31	4
		Nucleofection	pRC/RSV HC				
IgG 2G12	Anti-HIV IgG	Lipofection	pRC/RSV LC	1.0	4	21	10
			pRC/RSV HC				
IgG 4E10	Anti-HIV IgG	Lipofection	pCI neo CMV LC	0.4	2	18	15
			pCI neo CMV HC				
Fab 3H6	Fab Fragments	Protein-free	pIRES CMV HC LC	1.5	2	24	10
		Nucleofection	pIRES CMV LC dhfr				
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 $2\times$ SSC pH 7.0/50% Formamide at 45°C , then 5 times for 2 min $2\times$ 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 ($0.5\text{ }\mu\text{g ml}^{-1}$, 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} $\text{Na}_2\text{HPO}_4\cdot 2\text{H}_2\text{O}$), 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 $q_{\text{cDNA/RNA}}$ 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, $5\times$ 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 LumiImagerTM (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 meta-

centric 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 *dhfr* 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, homogeneously 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 con-

tent 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

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/*EcoRI* + *HindIII* 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

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.

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 μ M MTX in case of HGP and 1.0 μ M 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.

Acknowledgements This research was kindly funded by ACBT (Austrian Center of Biopharmaceutical Technology), a competence centre supported by the Federal Ministry of Economy and Labour and the federal states of Vienna and Tyrol. The authors are grateful to Annalisa Lasagna for analysis of product concentrations and to Friedemann Hesse for carefully reviewing the manuscript.

References

- Barnes LM, Bentley CM, Dickson AJ (2002) Stability of protein production from recombinant mammalian cells. *Biotech Bioeng* 81:631–639
- Barnett RS, Limoli KL, Huynh TB, Ople EA, Reff ME (1995) Antibody production in Chinese Hamster ovary cells using an impaired selectable marker. In: Wang HY, Imanaka T (eds) *Antibody expression and engineering*. American Chemical Society, Washington, DC
- Brown PC, Beverley SM, Schimke RT (1981) Relationship of amplified dihydrofolate reductase genes to double minute chromosomes in unstably resistant mouse fibroblast cell lines. *Mol Cell Biol* 1(12):1077–1083
- Chen C, Chasin LA (1998) Cointegration of DNA molecules introduced into mammalian cells by electroporation. *Somatic Cell Mol Genetics* 24(4):249–256
- Davies J, Reff M (2001) Chromosome localization and gen-copy-number quantification of three random integrations in Chinese-hamster ovary cells and their amplified cell lines using fluorescence in situ hybridisation. *Biotechnol Appl Biochem* 33:99–105
- Derouazi M, Martinet D, Besuchet Schmutz N, Flaction R, Wicht M, Bertschinger M, Hacker DL, Beckmann JS, Wurm FM (2006) Genetic characterization of CHO production host DG44 and derivative recombinant cell lines. *Biochem Biophys Res Commun* 340(4):1069–1077. Epub 2005 Dec 27
- Hoess RH, Ziese M, Sternberg N (1982) P1 site-specific recombination: nucleotide sequence of the recombining sites. *Proc Natl Acad Sci USA* 79(11):3398–3402
- Kao FT, Puck TT (1968) Genetics of somatic mammalian cells, VII. Induction and isolation of nutritional mutants in Chinese hamster cells. *Proc Natl Acad Sci USA* 60(4):1275–1281
- Kaufman RJ, Brown PC, Schimke RT (1979) Amplified dihydrofolate reductase genes in unstably methotrexate-resistant cells are associated with double minute chromosomes. *Proc Natl Acad Sci USA* 76:5669–5673
- Kaufman RJ, Sharp PA, Latt SA (1983) Evolution of chromosomal regions containing transfected and amplified dihydrofolate reductase sequences. *Mol Cell Biol* 3(4):699–711
- Kaufman RJ, Wasley LC, Spiliotes AJ, Gossels SD, Latt SA, Larsen GR, Kay RM (1985) Coamplification and coexpression of human tissue-type plasminogen activator and murine dihydrofolate reductase sequences in Chinese hamster ovary cells. *Mol Cell Biol* 5(7):1750–1759
- Kim SJ, Lee GM (1999) Cytogenetic analysis of chimeric antibody-producing CHO cells in the course of dihydrofolate reductase-mediated gene amplification and their stability in the absence of selective pressure. *Biotech Bioeng* 64(6):741–749
- Kuo MT, Vyas RC, Jiang LX, Hittelman WN (1994) Chromosome breakage at a major fragile site associated with P-glycoprotein gene amplification in multi-drug-resistant CHO cells. *Mol Cell Biol* 14(8):5202–5211
- Nunberg JH, Kaufman RJ, Schimke RT, Urlaub G, Chasin LA (1978) Amplified dihydrofolate reductase genes are localized to a homogeneously staining region of a single chromosome in a methotrexate-resistant Chinese hamster ovary cell line. *Proc Natl Acad Sci USA* 75(11):5553–5556

- O’Gorman S, Fox DT, Wahl GM (1991) Recombinase-mediated gene activation and site-specific integration in mammalian cells. *Science* 251(4999):1351–1355
- Palin AH, Critcher R, Fitzgerald DJ, Anderson JN, Farr CJ (1998) Direct cloning and analysis of DNA sequences from a region of the Chinese hamster genome associated with aphidicolin-sensitive fragility. *J Cell Sci* 111:1623–1634
- Pallavicini MG, De Teresa PS, Rosette C, Gray JW, Wurm FM (1990) Effects of methotrexate on transfected DNA stability in mammalian cells. *Mol Cell Biol* 10(1):401–404
- Ray M, Mohandas T (1976) Proposed banding nomenclature for the Chinese hamster chromosomes (*Cricetus griseus*). *Cytogenet Cell Genet* 16(1–5):83–91
- Strutzenberger K, Borth N, Kunert R, Steinfeldner W, Katinger H (1999) Changes during subclone development and ageing of human antibody-producing recombinant CHO cells. *J Biotechnol* 69:215–226
- Urlaub G, Chasin LA (1980) Isolation of Chinese hamster cell mutants deficient in dihydrofolate reductase activity. *Proc Natl Acad Sci USA* 77(7):4216–4220
- Varshavsky A (1981) Phorbol ester dramatically increases incidence of methotrexate-resistant mouse cells: possible mechanisms and relevance to tumor promotion. *Cell* 25(2):561–572
- Wahl GM, Vitto L, Padgett RA, Stark GR (1982) Single-copy and amplified CAD genes in syrian hamster chromosomes localized by a highly sensitive method for in situ hybridization. *Mol Cell Biol* 2(3):308–319
- Wilson C, Bellen HJ, Gehring WJ (1990) Position effects on eukaryotic gene expression. *Annu Rev Cell Biol* 6:679–714
- Yoshikawa T, Nakanishi F, Itami, S, Kameoka D, Omasa T, Katakura Y, Kishimoto M, Suga K-I (2000a) Evaluation of stable and highly productive gene amplified CHO cell line based on the location of amplified genes. *Cytotechnology* 33:37–46
- Yoshikawa T, Nakanishi F, Ogura Y, Oi D, Omasa T, Katakura Y, Kishimoto M, Suga K-I (2000b) Amplified gene location in chromosomal DNA affected recombinant protein production and stability of amplified genes. *Biotechn Prog* 16:710–715