Evaluation of stable and highly productive gene amplified CHO cell line based on the location of amplified genes

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Abstract

In order to establish an easy and quick construction method for obtaining a stable and highly productive gene-amplified recombinant Chinese Hamster Ovary (CHO) cell line, various kinds of stepwise methotrexate (MTX) selection were carried out. The specific growth and production rates of the cell were compared with each other, and the distribution of the amplified gene location was determined using fluorescence *in situ* hybridization (FISH). The specific growth and production rates of the cell pool reached the highest levels under the selection condition in which the stepwise increase in the MTX concentration was most gradual; about 82% of amplified genes were observed near the telomeric region. During long-term cultivation without MTX, the percentage of amplified genes near the telomeric region hardly changed, but that of amplified genes at other regions decreased. Based on these results, stable and highly productive cell pools could be easily and quickly constructed and amplified and gradual stepwise increase of the MTX concentration. In addition, the FISH technique was powerful tool to evaluate highly productive and stable gene-amplified cells based on the chromosomal location of the amplified gene.

Abbreviations: CHO, chinese hamster ovary; *dhfr*, dihydrofolate reductase; hGM-CSF, human granulocyte macrophage colony stimulating factor; FISH, fluorescence *in situ* hybridization; MTX, methotrexate.

Introduction

The amplified genes are usually located either within expanded chromosomal regions, termed homogeneously staining regions (HSRs), in abnormally banded regions (ABRs), or in extrachromosomal elements termed double minutes (DM) (Qumsiten et al., 1993; Misawa et al., 1987). Some molecular biological mechanism for gene amplification have been proposed, for example unequal sister chromatid exchange, telomeric fusions and bridge-breakage-fusion cycles, centromere recombination, and chromosome breakage-acentric element models (Kaufman, 1993; Mariani et al., 1984; Smith et al., 1990; Stark, 1993; Von Hoff, 1991; Windle et al., 1992). However,

the relationship between the selection methods and the gene amplification mechanisms remains open to investigation.

In general, MTX-tolerant cell lines develop resistance against MTX owing to *dhfr* gene amplification. Other mechanisms of tolerance to MTX have been reported, such as the membrane-permeability mutation (Assaraf and Schimke, 1987), the expression of DHFR, with no- or low sensitivity to MTX (Haber et al., 1981). The appearance of these other tolerant cells might correspond to the increase method of MTX concentration.

Gene amplification techniques using recombinant mammalian cells are frequently employed for the production of glycoproteins (Cockett et al., 1990; Kane

Figure 1. Construction of dhfr and hGM-CSF gene amplification vector. The pSV2-dhfr/hGM-CSF vector was constructed from pSV2-dhfr and pcD-hGM-CSF. The pSV2-dhfr vector contained dhfr cDNA derived from mouse and the pcD-hGM-CSF vector contained human GM-CSF gene.

et al., 1988; Kaufman et al., 1985; Kaufman, 1993), among which the dihydrofolate reductase (*dhfr*) gene amplification system in the Chinese Hamster Ovary (CHO) cell lines are the most widely used (Alt et al., 1978; Kaufman, 1993). In regard to the application of a gene amplification system for industrial processes, one of the most important factors is the selection method employed for obtaining highly productive recombinant CHO cell lines that can stably produce the desired recombinant proteins (Kaufman et al., 1982; Pallavicini et al., 1990; Rath et al., 1984; Weidle et al., 1988). However, the selection has so far been carried out only empirically.

Rath et al. (1984) investigated the time required for mouse 3T6 cells to develop resistance to 200 nM methotrexate (MTX) based on three selection protocols, and found that multistep selection was the most effective for rapid emergence of resistance. In single-step (rapid) selection, gene-amplified cell lines were not obtained, whereas in two or greater number of step selections, amplified *dhfr* genes were obtained. However, in these experiments, stability of the amplified genes in the absence of MTX was not evaluated. Using mammalian cells including CHO cells, Kaufman et al.

(1985) and Pallavicini et al. (1990) examined the stability of amplified gene in the presence and absence of MTX (Pallavicini et al., 1990; Weidle et al., 1988), and concluded that the amplified genes were more stable when the cells were cultured with MTX.

In order to obtain a 'gene-amplified' cell pools, recombinant cell pools were cultivated in a selection medium containing MTX, which inhibits DHFR. With increase in MTX concentration most of the cells died, but a proportion in which *dhfr* was amplified, was able to survive in the medium containing MTX. In order to systematically construct highly productive cell pools, an optimal strategy for stepwise selection must be devised. Therefore, we carried out stepwise increase in MTX concentration under five different selection conditions (pattern 1 to 5 in Figure 2a) (Omasa et al., 1996). Therefore, in order to obtain a highly productive and stable cell, systematic evaluation of methods for the selection of a stable and highly productive cell is essential. We previously reported that the specific growth and production rates were affected by stepwise selection patterns (Omasa et al., 1996). In this experiment, we investigated the effect of the pattern of increase of the MTX concentration on the specific

Figure 2. Relationship between pattern of increase in MTX concentration and specific growth and production rates and stepwise selection. The calculations of the specific growth and production rates are based on the use of heterogeneous cell lines. This calculation procedure is described in the Experimental protocol. In the 1000 nM-MTX condition, we did not obtain tolerant cell lines (data not shown). (a) Stepwise selection patterns. (b) Specific human GM-CSF production rate (top); specific growth rate (bottom).

production rate, the distribution of location and stability of the amplified genes, for the selection of a stable and highly productive cell.

MTX addition to the culture medium in a *dhfr* amplification system is frequently employed to in-

duce gene amplification (Rath et al., 1984). For the industrial production of recombinant proteins, cultivation without MTX has been adopted, because MTX is a toxic drug. However, during long-term cultivation without MTX, amplified genes were gradually deleted

and the recombinant protein production rate also decreased (Pallavicini et al., 1990; Rath et al., 1984). Therefore, a suitable method for the construction of stable and highly productive cell lines was sought for the production of recombinant proteins. We analyzed the stability of amplified genes during long-term cultivation with and without MTX, using the technique of fluorescence *in situ* hybridization (FISH).

Materials and methods

Vectors and transfection

The pSV2-dhfr/hGM-CSF vector (Figure 1) was constructed from pSV2-dhfr (ATCC 37146) (Southern and Berg, 1982; Subramani et al., 1981) and pcD-hGM-CSF (ATCC 57594) (Lee et al., 1985; Okayama and Berg, 1983). The pSV2-dhfr vector contained dhfr gene derived from mouse and the pcD-hGM-CSF vector contained human GM-CSF gene. The promoter for both vectors was SV40 early promoter (Fiers et al., 1978; Reddy et al., 1978) and the terminator, SV40-polyA, was derived from SV40. These vectors ($40 \mu\text{g ml}^{-1}$) were introduced into host cells (5×10^7 cells ml^{-1}) using an electroporation procedure (Shimadzu SSH-10, PW = 50 μs , PH(VDC) = 600 kV, PULCE n = 2, RPT INTTVL = 1.0 s) (Potter et al., 1993).

Cell line and culture conditions

The CHO DG44 cell line (*dhfr*⁻), which was kindly provided by Dr. L. Chasin of the Columbia University, was used as the host cell line. The host cells were maintained in Iscove's modified DMEM (Sigma I-7633) supplemented with 10% fetal bovine serum (FBS; Gibco 26140-020), hypoxanthine (13.6 mg l^{-1}), and thymidine (2.42 mg l^{-1}). Transformants (*dhfr*⁺; derived from DG44) were cultivated in the selection medium Iscove's modified DMEM supplemented with 10% dialyzed FBS and different concentrations of MTX. For dhfr gene amplification, 50 to 1000 nM (final concentrations) of methotrexate (MTX; Sigma A6770 (+) Amethopterin) were added to the selection medium according to one of five patterns (Figure 2a). Cells were incubated in 5% of CO₂ at 37 °C.

Calculation of specific growth and hGM-CSF production rates

Cell samples (1 Vol.) were diluted with 0.16% Trypan blue -0.85% NaCl solution, which stained only dead cells. The viable cell number was measured using a Bürker-Türk hemacytometer (ERMA 4296). This measurement method was described in detail by Omasa et al. (1992). The specific growth rate based on viable cells was calculated using the following equations (Equations (1) and (2)).

$$\frac{dX_t}{dt} = \mu X_v \quad (1)$$

$$\ln(X_t) = \mu \int \left(\frac{X_v}{X_t} \right) dt + \ln(X_t)_0 \quad (2)$$

The hGM-CSF concentration was measured by using a sandwich Enzyme-Linked Immunosorbent Assay (ELISA) using 96-well plates (Greiner 655061). The 96-well plate was previously coated with sheep anti-hGM-CSF antibody (580 ng well^{-1}) (Endrogen P521) over night at 4 °C. One hundred μl of culture supernatant was applied to each well and plates were incubated for 2 h at 37 °C. Polyclonal rabbit anti-hGM-CSF antibody (Genzyme LP-714) was applied as the primary antibody (0.2 $\mu\text{g well}^{-1}$) and plates were incubated for 2 h at 37 °C. Alkaline phosphatase-conjugated anti-rabbit IgG antibody (Tago ALI3405) was used as the secondary antibody (0.1 $\mu\text{g well}^{-1}$) and plates were incubated for 2 h at 37 °C. After incubation, *p*-nitrophenyl phosphate (pNPP) (Sigma N-9389) was used as a substrate for alkaline phosphatase (0.2 mg well^{-1}) and enzyme reaction was carried out for 1 h at 37 °C. Purified recombinant hGM-CSF (Genzyme RH-CSF-C) was used as a standard. The absorbance of the sample was measured at 405 nm using an ELISA microplate reader (Tosoh MPR A4). The specific hGM-CSF production rate was calculated using the following equations (Equations (3) and (4)):

$$\frac{dP}{dt} = \rho X_v \quad (3)$$

$$P = \rho \int X_v dt + P_0 \quad (4)$$

The change of cell numbers and human GM-CSF concentration were measured by short batch cultures (4 days) using the constructed cell lines. The specific growth and production rates were estimated with 95% confidential level based on Student's *t* distribution (Chatterlee et al., 1991).

Group T... telomeric region

Group O... other regions

Group N... no signal

Figure 3. Classification of amplified gene location on chromosome using FISH. The procedure for FISH procedure was described in the Experimental protocol. Chromosomes were stained with DAPI and green signals (FITC) on chromosomal amplified gene locations. Amplified genes located near the telomeric region were classified as Group T (right upper picture) and those located in other chromosomal regions were classified as Group O (right middle picture). Those emitting no signals were classified as Group N (right lower picture).

Figure 4. Relationship between pattern of increase in MTX and amplified gene locations. The procedure for FISH was described in the Experimental protocol. The percentage of amplified genes was calculated from the observation of the amplified gene locations in 100 chromosomal DNA sets per cell line. All cell lines, patterns 1 to 5, exhibited tolerance to 1000 nM-MTX and were derived from stepwise selections as shown in Figure 1a.

Fluorescence in situ hybridization (FISH)

Chromosome spreads were prepared from exponential phase cultures using standard techniques (Carroll et al., 1988). The cells were treated with colcemid ($10 \mu\text{g ml}^{-1}$ -medium) for 5 to 6 h at 37°C , followed by hypotonic solution (1.5 ml of 75 mM KCl) for 20 min at room temperature. After the KCl was decanted, freshly made fixative (3:1 v/v, methanol/acetic acid) was added. This procedure was repeated 3 times, and the suspension was dropped on slides (Preclean; Matsunami S-2124) (Okumura, 1992; Kuriki et al., 1993).

Fluorescence *in situ* hybridization (FISH) was performed essentially as described by Pinkel et al. (1986). Fixed chromosomal DNA was denatured in 70% formamide and $2\times$ standard saline citrate (SSC) at 70°C ($\pm 2^\circ\text{C}$) for 2 min. The *dhfr* probe was biotinylated by nick-translation with biotin-16-dUTP (Boehringer Mannheim 1093070). The hybridization mixture was incubated for 2 h at 15°C and denatured for 10 min at 100°C . Using agarose-gel electrophoresis, the probe

length was checked to be approximately 200–300 bp. Chromosomal DNA was washed in 70, 90 and 100% ethanol solution for 5 min, three times. The probe was denatured for 10 min at 80°C , and mounted on glass slides shielded by paper bond. Hybridization was carried out overnight at 37°C . The slide glass was washed strictly at 43°C in 50% formamide, $2\times$ SSC three times and in $2\times$ SSC at 60°C three times. Blocking reagent (3% (w/v) Block Ace; Yukijirushi Co. Cat No.UK-B80, $4\times$ SSC, 0.1% (v/v) Tween 20) was mounted on the slide. The slide was incubated for 30 min at 37°C . The probe was detected using fluorescein isothiocyanate (FITC)-labeled streptavidin. The chromosomes were stained with 4,6-diamidino-2-phenylindole (DAPI) and observed under a Zeiss Axioplan microscope. Photographs were taken with a cooled CCD camera (IPLab. SpectrumTM controlled Photometrics PVCAM camera system). The chromosome image was captured in monochrome 256 gradation and was painted and modified using Adobe Photo-Shop 4.0J. On the basis of the observations, the amplified genes were classified into three groups (telomere type, other types, and those with no signal).

Results and discussion

In order to systematically construct a highly productive cell pool, an optimal strategy for stepwise selection must be devised. Therefore, we carried out stepwise increase in MTX concentration under five different selection conditions (pattern 1 to 5 in Figure 2a) (Omasa et al., 1996). We have already carried out five stepwise increases in MTX concentration with an initial MTX concentration of 10 nM (Omasa et al., 1996). In the case of the 10 nM initial MTX concentration patterns, the tendency for gene amplification was almost the same as for the 0 nM initial MTX concentration patterns (data not shown). At initial MTX concentration over 1000 nM, no MTX-tolerant cell lines were obtained. Under the five different selection conditions used (pattern 1 to 5 in Figure 2a), five cell pools (named DR1000L1 to DR1000L5) were obtained.

The specific growth (μ) and human GM-CSF production (ρ) rates in these cell pools were calculated at each stepwise increase in MTX concentration (Figure 2b). The results revealed that the DR1000L1, L2 and L3 cell pools, cultivated in the presence of gradually increasing MTX concentrations, exhibited higher specific production rates than the other cell pools (DR1000L4 and L5) (Figure 2b).

Figure 5. Changes in the specific growth (μ) and specific production (ρ) rates during long-term cultivation using the DR1000L1 cell line. The DR1000L1 cell line was used for this cultivation. For the 1000 nM-MTX condition, MTX was added to Iscove's modified DMEM medium with 10% dialyzed FBS. Calculation methods for specific growth and hGM-CSF production rates were described in the Experimental protocol. Closed and open circles denote cultivation condition with and without MTX, respectively.

Figure 6. Changes in the locations of amplified genes during long-term cultivation using the DR1000L1 cell line. The FISH procedure was described in the Experimental protocol. The percentage of amplified genes was calculated from the observation of the amplified gene locations in 100 chromosomal DNA sets per cell line. (a) Long-term cultivation with MTX (1000 nM). (b) Long-term cultivation without MTX.

Several molecular biological mechanisms for gene amplification have been proposed (Stark, 1993; Windle and Wahl, 1992). Some investigators have proposed dhfr gene amplification model in CHO cells, being located near the telomeric region (Kaufman et al., 1983; Ruiz and Wahl, 1990). Others have also reported the amplified genes as being located near the centromeric region (Pallavinici et al., 1989, 1990). At telomeric sites, gene amplification occurred by fusion between two telomere regions. At non-telomeric sites such as centromeric sites replication and sister-chromatid exchange models were proposed (Morgan et al., 1986; Barclay et al., 1982). When amplified genes were integrated into different chromosomal DNA positions, we suspected that these differences in the location of amplified gene affected the gene amplification efficiency during stepwise selection. Following stepwise selection, the amplified gene copy number changed. We speculate that the differences in specific production rates depend on the amplified gene location on the chromosomal DNA.

In order to detect the location of the amplified gene on chromosomal DNA, the FISH technique was applied to the gene-amplified cell pools DR1000L1 to L5. We collected cells in the mitotic period and observed the amplified gene locations against 100 chromosomal DNA sets per cell pools. On the basis of such observations, the amplified genes were classified into three groups: the amplified genes which were located in the telomeric regions (Figure 3a), amplified genes located in other regions (Figure 3b), and genes not emitting FISH signals on the chromosomal DNA (Figure 3c). In some chromosomal DNA sets of the cells, we were not able to classify the amplification type, but these chromosomal DNA sets were quite rare (data not shown). We were safely able to neglect such chromosomal DNAs in this classification.

Using this classification, we determined the distribution of amplified gene locations (Figure 4) in five cell pools (Figure 2a). The distribution of amplified gene locations was found to depend on the pattern of increase in MTX concentration as shown in Figure 4. When the stepwise increase in the MTX concentration was most gradual, 82% of the amplified genes in the cell pools (DR1000L1 (Figure 2b)) were observed near the telomeric region. With a relatively more rapid increase in MTX concentration (DR1000L4 and 5), the percentage of amplified genes near the telomeric region decreased and no signal increase was observed in comparison with DR1000L1.

MTX addition was necessary to induce dhfr gene amplification. Generally, selection pressure is necessary to maintain stable production of recombinant protein in mammalian cells, including CHO cells (Pallavinici et al., 1990; Weidle et al., 1988). However, because liver and kidney lesions have been induced as a result of MTX administration to patients, MTX elimination from the cultivation medium is desirable for human GM-CSF production. However, during long-term cultivation without MTX, the amplified genes were gradually deleted and in parallel the specific recombinant proteins production rate decreased (Pallavinici et al., 1990; Rath et al., 1984).

In order to evaluate the relationship between the stability of the amplified genes and their locations, we carried out long-term cultivation with and without MTX by using the DR1000L1 cell pool. During cultivation with MTX, the specific growth and production rates remained constant. While, during cultivation without MTX after 46 days, the specific human GM-CSF production rate decreased by approximately 30% compared with the initial rate as shown in Figure 5. Two reasons are probable for this decrease. One reason is the increase of low productive cells, such as no signal type. The second reason is that the other type cells were not so stable compared with the telomere type cells in the conditions without MTX. Figure 6 shows the changes in the location of amplified genes during long-term cultivation. During long-term cultivation with MTX, the profiles of amplified genes did not change (Figure 6a). Without MTX, however, the profile of amplified genes near the telomeric region scarcely changed, while the percentage of genes emitting no signals gradually increased, along with a decrease in the percentage of amplified genes situated in other regions (Figure 6b). These results reveal that during long-term cultivation, amplified genes, which are located near the telomeric region, are more stable than those located in other regions.

Our results in this study demonstrated that the amplified gene location is closely correlated to the expression of amplified genes and to the amplified gene deletion from chromosomal DNA. We speculated that the cells that had amplified genes near the telomeric region might have a higher specific production rate compared to other types of gene-amplified cells. From our results shown in Figures 2b and 4, the DR1000L1, with a great number of amplified genes located near the telomeric region, had the highest specific production rate. Therefore, in order to construct highly

productive cells, gradual stepwise selection is more desirable than rapid stepwise selection.

We are now planning to investigate the relationship between the stability and locations of the amplified genes in detail by using clones of both telomeric and other types of amplified genes.

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Nomenclature

- X_t : total cell number ($\text{cell} \cdot \text{l}^{-1}$).
 X_v : living cell number ($\text{cell} \cdot \text{l}^{-1}$).
 P : human GM-CSF concentration ($\text{g} \cdot \text{l}^{-1}$).
 μ : specific growth rate (h^{-1}).
 ρ : specific human GM-CSF production rate ($\text{g} \cdot \text{cell}^{-1} \cdot \text{l}^{-1}$).

References

- Alt FW, Kellems RE, Bertino JR and Schimke RT (1978) Selective multiplication of dihydrofolate reductase genes in methotrexate-resistant variants of cultured murine cells. *J Biol Chem* 253: 1357–1370.
- Assaraf YG and Schimke RT (1987) Identification of methotrexate transport deficiency in mammalian cells using fluoresceinated methotrexate and flow cytometry. *Proc Natl Acad Sci USA* 84: 7154–7158.
- Barclay BJ, Kunz BA, Little JG and Haynes RH (1982) Genetic and biochemical consequences of thymidylate stress. *Can J Biochem* 60: 172–184.
- Carroll SM, DeRose ML, Gaudray P, Moore CM, Needham-Vandevanter DR, von Hoff DD and Wahl GM (1988) Double minute chromosomes can be produced from precursors derived from a chromosomal deletion. *Mol Cell Biol* 8: 1525–1533.
- Chatterjee S and Price B (1991) Regression analysis by example. A Wiley-Interscience publication, pp. 1–21, New York, USA.
- Cockett MI, Bebbington CR and Yarranton GT (1990) High level expression of tissue inhibitor of metalloproteinases in chinese hamster ovary cells using glutamine synthetase gene amplification. *Bio/Technology* 8: 662–667.
- Fiers W, Couterras R, Haegemann G, Rogiers R, van de Voorde A, van Heuverswyn H, van Herreweghe J, Volckaert G and Ysebaert M (1978) Complete nucleotide sequence of SV40 DNA. *Nature* 273: 113–120.
- Haber DA, Beverley SM, Kiely ML and Schimke RT (1981) Properties of an altered dihydrofolate reductase encoded by amplified genes in cultured mouse fibroblasts. *J Biol Chem* 256: 9501–9510.
- Ichikawa D, Hashimoto N, Hoshima M, Yamaguchi T, Sawai K, Nakamura Y, Takahashi T, Abe T and Inazawa J (1996) Analysis of numerical aberrations of specific chromosomes by fluorescence *in situ* hybridization as a diagnostic tool in breast cancer. *Cancer* 77: 2064–2069.
- Kane SE, Troen BR, Gal S, Ueda K, Pastan I and Gottesman MM (1988) Use of a cloned multidrug resistance gene for coamplification and overproduction of major excreted protein, a transformation-regulated secreted acid protease. *Mol Cell Biol* 8: 3316–3321.
- Kaufman RJ and Sharp PA (1982) Amplification and expression of sequences cotransfected with a modular dihydrofolate reductase complementary DNA gene. *J Mol Biol* 159: 601–621.
- Kaufman RJ, Sharp PA and Latt SA (1983) Evolution of chromosomal regions containing transfected and amplified dihydrofolate reductase sequences. *Mol Cell Biol* 3: 699–711.
- Kaufman RJ, Wasley LC, Spiliotes AJ, Gossels SD, Latt SA, Larsen GR and 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: 1750–1759.
- Kaufman RJ (1993) Amplification and expression of transfected genes in mammalian cells. In: Kellems RE (ed) *Gene Amplification in Mammalian Cells* (pp. 315–343). Marcel Dekker Inc., New York.
- Kuriki H, Sonta S and Murata K (1993) Flow karyotyping analysis and sorting of the chinese hamster chromosomes: comparing the effects of the isolation buffers. *J Clin Lab Anal* 7: 119–122.
- Lee F, Yokota T, Otsuka T, Gemmell L, Larson N, Luh J, Arai K and Rennick (1985) Isolation of cDNA for a human granulocyte-macrophage colony-stimulating factor by functional expression in mammalian cells. *Proc Natl Acad Sci USA* 82: 4360–4364.
- Mariani BD and Schimke RT (1984) Gene amplification in a single cell cycle in chinese hamster ovary cells. *J Biol Chem* 259: 1901–1910.
- Misawa S, Staal SP and Testa JR (1987) Amplification of the c-myc oncogene is associated with an abnormally banded region on chromosome 8 or double minute chromosomes in two HL-60 human leukemia sublines. *Cancer Genet Cytogenet* 28: 127–135.
- Morgan WF, Bodycot J, Fero ML, Hahn PJ, Kapp LN, Pantelias GE and Painter RB (1986) A cytogenetic investigation of DNA replication after hydroxyurea treatment: implications for gene amplification. *Chromosoma* 93: 191–196.
- Okayama H and Berg P (1983) A cDNA cloning vector that permits expression of cDNA inserts in mammalian cells. *Mol Cell Biol* 3: 280–289.
- Okumura K (1992) Analysis of DNA in interphase nuclei by fluorescence *in situ* hybridization. *Tanpakushitsu Kakusan Koso* 37: 1546–1554.
- Omasa T, Higashiyama K, Shioya S, Suga K (1992) Effects of lactate concentrations on hybridoma culture in lactate-controlled fed-batch operation. *Biotechnol Bioeng* 39: 556–564.
- Omasa T, Itami S, Kameoka D, Katakura Y and Suga K (1996) Selection and stability for recombinant CHO cell line expressing human GM-CSF in gene amplification. Nagai K and Wachi M (eds), *Animal Cell Technology: Basic and Applied Aspects* (pp. 29–33). Kluwer Academic Publishers, The Netherlands.
- Pallavicini MG, DeTeresa PS and Wurm FM (1989) Use of fluorescence *in situ* hybridization to detect and monitor transfected and

- amplified sequences in recombinant CHO cells. *Dev Biol Stand* 70: 165–172.
- Pallavicini MG, DeTeresa PS, Rosette C, Gray JW and Wurm FM (1990) Effects of methotrexate on transfected DNA stability in mammalian cells. *Mol Cell Biol* 10: 401–404.
- Pinkel D, Straume T and Gray JW (1986) Cytogenetic analysis using quantitative, high-sensitivity, fluorescence hybridization. *Proc Natl Acad Sci USA* 83: 2934–2938.
- Potter H, Weir L and Leder P (1984) Enhancer-dependent expression of human κ immunoglobulin genes introduced into mouse pre-B lymphocytes by electroporation. *Proc Natl Acad Sci USA* 81: 7161–7165.
- Qumsiyen MB, Goorha S and Suttle DP (1993) Gene amplification and chromosome rearrangements: A study of a single cell lineage selected for amplification and deamplification of the UMP synthase gene. *Cytogenet Cell Genet* 62: 162–168.
- Rath H, Tlsty T and Schimke RT (1984) Rapid emergence of methotrexate resistance in cultured mouse cells. *Cancer Res* 44: 3303–3306.
- Reddy VB, Thimmappaya B, Dhar R, Subramanian KN, Zain BS, Pan J, Ghosh PK, Celma ML and Weissman SM (1978) The genome of simian virus 40. *Science* 200: 494–502.
- Ruiz JC and Wahl GM (1990) Chromosomal destabilization during gene amplification. *Mol Cell Biol* 10: 3056–3066.
- Selig S, Okumura K, Ward DC and Cedar H (1992) Delineation of DNA replication time zones by fluorescence *in situ* hybridization. *EMBO J* 11: 1217–1225.
- Smith KA, Gorman PA, Stark MB, Groves RP and Stark GR (1990) Distinctive chromosomal structures are formed very early in the amplification of CAD genes in syrian hamster cells. *Cell* 63: 1219–1227.
- Southern PJ and Berg P (1982) Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. *J Mol Appl Genet* 1: 327–341.
- Stark GR (1993) Regulation and mechanisms of mammalian gene amplification. *Adv Cancer Res* 61: 87–113.
- Subramani S, Mulligan R and Berg P (1981) Expression of the mouse dihydrofolate reductase complementary deoxyribonucleic acid in simian virus 40 vectors. *Mol Cell Biol* 1: 854–864.
- Von Hoff DD (1991) New mechanisms of gene amplification in drug resistance (the episome model). In: Robbert F. Ozols (ed) *Molecular and Clinical Advances in Anticancer Drug Resistance*. Cancer Treatment and Research Vol. 57 (pp. 1–11). Kluwer Academic Publishers, The Netherlands.
- Weidle UH, Buckel P and Wienberg J (1988) Amplified expression constructs for human tissue-type plasminogen activator in chinese hamster ovary cells: instability in the absence of selective pressure. *Gene* 66: 193–203.
- Windle BE and Wahl GM (1992) Molecular dissection of mammalian gene amplification: new mechanistic insights revealed by analyses of very early events. *Mutat Res* 276: 199–224.