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The Chromosomal DNA Position of Amplified Genes Affects Recombinant Protein Production and Gene Stability

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Abstract :

Previously, we developed a simple and rapid approach for generating a stable and highly prolific CHO cell line that has been amplified using genes. Increased concentrations of methotrexate (MTX) resulted in high and consistent specific growth and production rates for gene-amplified cell populations. Gene-amplified cells' phenotypic seemed to be influenced by the chromosomal DNA region in which the amplified gene was located. Over the course of our long-term selection process, we hypothesized that a variety of gene-amplified cells would emerge. We extracted gene-amplified clones obtained from gene-amplified cell pools to better understand how gene-amplified cell pools respond to a stepwise rise in MTX concentration. Recombinant protein production, the stability of amplified genes, the location of amplified genes were compared across isolated clones. This led to the discovery of more stable and prolific "telomeretype clones," which had an amplified gene situated near the telomeric region. The chromosomal DNA of telomere-type clones included over 100 copies of amplified genes. It's not uncommon to see fewer than 10 copies of amplified genes in a big number of additional clones. While in the absence of MTX, in other kinds of clones, amplified genes quickly depleted from chromosomal DNA after prolonged culture.

Introduction

eukaryotes have an abundance of genes that may be repurposed for many purposes. 1-3, the formation of drug resistance in tumor cells and certain human parasites (4, 5) and the maturation of cancer cells are all examples of this process (4, 5). Furthermore, the gene amplification phenomenon has been used to produce recombinant drugs by employing cultivated cells (6, 7). Until today, the mechanics of gene amplification were completely unknown. Various host cells, including E. coli, yeast, and mammalian cells, have been employed in commercial operations to create recombinant medicines. EPO, granulocyte colony-stimulating factor (G-CSF), and other antibodies have been produced in commercial procedures using recombinant expression systems (6, 7). Recombinant protein manufacturing employing mammalian cell lines has yet to be fully established in an industrial setting. General methods for the development of productive cell lines have not yet been discovered as well. There was also no mention of alternative effective, rapid, and easy selection procedures, such as flow cytometry or cell affinity separtion. Gene amplification methods using recombinant mammalian cells are commonly used to increase the poor productivity of mammalian cells while producing glycoproteins (6-8). Dihydrofolate reductase (dhfr) gene amplification method in the Chinese hamster ovary is one of these gene amplification systems (CHO

Table 1. Rates of MTX Concentration Increase Depending on the Pattern of Increase

stepwise selection pattern	specific growth rate [h^{-1}]	specific production rate [10^{-16} g $cell^{-1}$ h^{-1}]
rapid increase mode[a]	0.019	1.0
gradual increase mode[b]	0.036	1.6

[a] 0 → 100 → 1000 nM MTX. [b] 0 → 50 → 200 → 500 → 1000 nM MTX.

in the majority of cases, the cell line (8, 9). Selection methods for recombinant CHO cell lines that can consistently generate the necessary recombinant proteins are critical for the use of gene amplification systems in industrial processes (10-12). However, thus far, the selection procedures have been based solely on trial and error and have been implemented experimentally. We're interested in how geneamplified cell pools behave throughout the course of long-term selection. Finally, we want to verify the quantitative selection approach in order to create highly productive and stable gene-amplified CHO cell lines that are long-lasting. Stepwise MTX concentration rise has recently been developed in CHO cells. For cell lines with high specific growth and production rates, a steady rise in MTX

concentration was more effective than a quick increase in MTX concentration (13, 14). (Table 1). We hypothesized that the stability and productivity of recombinant proteins are influenced by the cell line's gene-amplified cell line's location of amplified genes. Fluorescence in situ hybridization (FISH) was used to identify the locations of amplified genes on chromosomal DNA (FISH). Heterogeneous cell pools have been identified as a consequence of our analysis. The telomeric region of chromosomal DNA is home to several amplified genes. Genes that were amplified were found in less productive and more unstable heterogeneous cell pools, on the other hand. There were some cells that developed resistance while others perished throughout the process of stepwise selection of MTX-resistant cells. Cells with changed membrane permeability and alterations in the DHFR affinity for MTX survived the stepwise selection, for example, cells that were amplified genetically. However, it is still unknown how gene-amplified cell pools behave during selection. To better understand how gene-amplified cell pools behave during stepwise selection, we isolated distinct types of resistant cell clones and characterized their characteristics.. Quantitative evaluation and comparisons were conducted on the features of isolated clones, including rate of amplification, copy number of amplified genes, and protein production stability. This led to the discovery of high and stable recombinant protein production in certain cells with amplified genes situated near the telomeric region, as well as large copy numbers for the amplified genes. We used these findings to try to figure out how long-term culture of heterogeneous gene-amplified cell pools behaves.

Methods and Substances The cell line, the culture conditions, the vectors, and the trans-ecution.

Host cells were provided by Dr. L. Chasin of Columbia University's CHO DG44 cell line (dhfr-). They were maintained alive in IMDM (SigmaAldrich Japan, I-7633) with 10% FBS, hypoxanthine
(13.6 mg L-1, Yamasa), and thymidine (2.42 mg L1, Yamasa). IMDM dialyzed with 10% FBS but neither hypoxanthine or thymidine was utilized to select dhfr+ (DG44-derived) transformants. It was preserved at 37 degrees Celsius in a 5 percent CO2 environment. For each of the five patterns, Methotrexate (MTX; Sigma-Aldrich Japan) was utilized in the selection medium (14). There were five distinct cell pools in the DR1000L-1 to L-5 heterogeneous cell pool. These cell pools had an MTX concentration of 1000 nM. There were several different cell pools in which telomeres and other cell lines were cloned utilizing a restricted dilution method. IMDM medium was used to thin cell suspensions to one per 200 L before distributing them onto 96-well plates for this experiment. Cloned cells were expanded to a cell density of 106 per milliliter of culture medium before being used in the study. Afterwards, they were culled and sold. These steps were done over the course of a month. ATCC 37146 and pcD-hGM-CSF were utilized to construct the pSV2-dhfr/hGM-CSF vector (17, 18). (Figure 1). The dhfr gene was inserted into three vectors: two pSV2-dhfr constructs and a pcD-hGM-CSF construct. There were two vectors that employed SV40-polyA as a terminator for both of the promoters. Calculation of Specific Growth and hGM-CSF Production Rates with Step-by-Step Instructions for Trans Fecation.

Staining only dead cells was achieved by diluting cell samples (1 vol) with 0.16 percent Trypan Blue0.85 percent NaCl solution. A Bu rker-Tu rk hemacytometer was used to determine the number of viable cells (ERMA Tokyo, 4296). Omasa et al. went into great length about this measuring procedure (20). For the purpose of assessing the productivity and stability of the built-in cell pools,

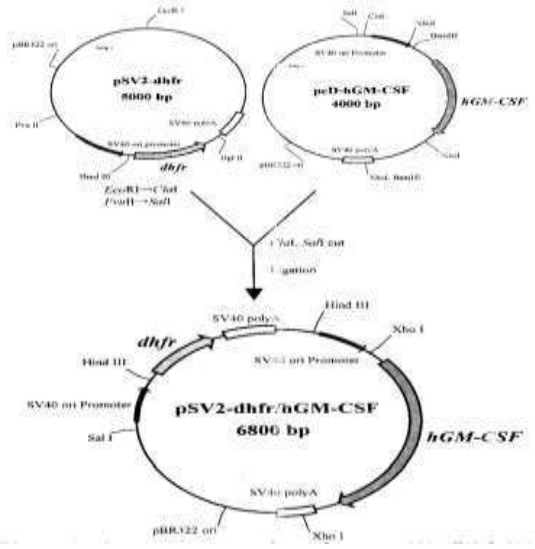


Figure 1. The process of creating the pSV2dhfr/hGM CSF vector. From the pSV2-dhfr and pcD-hGM-CSF vectors, a pSV2-dhfr/hGM-CSF vector was created There was a tandem arrangement of the Dhfr and hGM-CSF genes on this plasmid, and the SV40 ori promoter and SV40 polyA terminator were necessary to calculate the specific growth and human GM-CSF production rates (per cell per hour) with 95% confidence level. As a result of the findings, the level of confidence is less than 5% of the rates. It was determined using a 96-well plate enzyme-linked

immunosorbent assay (ELISA) (Greiner, 655061). By Yoshikawa et al., the ELISA approach and the computation of specific growth and production rates were explained in detail (14).

Copy Num-bers of dhfr and hGM-CSF

In order to determine the copy counts of the dhfr and hGM-CSF genes, we used the dot blotting approach (21). (Roche Diagnostics, 1093657). The DIG DNA labeling and detection kit's instruction booklet was followed closely for this particular testing process. In order to create two types of probes, restriction enzyme digestion was used (HinD III and Xho I, respectively). We used agarose gel electrophoresis to separate digested fragments and a prepA gene DNA purification kit to recover each fragment (Bio rad, 732-6010). Measurements of dhfr and CSF gene copy number were subject to experimental error. Experimental error was less than 10%. Hoechst 33258 was utilized as the DNA-binding specific reagent in a fluorometry measurement approach (22), which averaged the chromosomal DNA content. The AT-rich area of this reagent was particularly bound by this reagent, whereas RNA was not (23). In this experiment, the excited wavelength was 365 nm, while the emission wavelength was 455nm. Standard DNA concentration was -DNA (Takara, 3010).

Fluorescence Hybridization in the Field (FISH)

Standard methods were used to produce chromosome spreads from exponential phase cells (24). Colcemid (10 g mL-1-medium) was incubated with the cells for 5-6 hours at 37 °C. At room temperature, 1.5 mL of 75 mM KCl was used to suspend the cells for 20 minutes. Freshly produced fixative (3:1 MeOH/HOAc) was added after the hypotonic solution was decanted. Drops of the suspension were deposited on a slide (Preclean; Matsunami, S-2124) three times in a row (25, 26). FISH was carried out as reported by Pinkel et al. in this study (27). FISH's specifics have already been discussed (14). A distribution of amplified genes was derived from the findings by classifying the amplified genes into three categories (telomere-type, other kinds, and those without signals).

Measurement of DHFR Activity.

One million cells were collected and resuspended in 500 L of PBS (PSB, pH 7.4). Afterward, they were placed in a water bath at 4 degrees Celsius after being sonicated for 5 s. There were four instances of these operations being performed. By centrifuging at 18 000 g for 5 minutes, the crude enzyme was isolated. 340 nm absorbance at 30 degrees Celsius was used to test the absorbance of a solution consisting of a 200 nM KH2PO4/KCl buffer, 500 L of 500 M dihydro folic acid (D7006), 290 L of ultrapure water, 10 mM â-NADPH, and 100 L of crude enzyme solution.

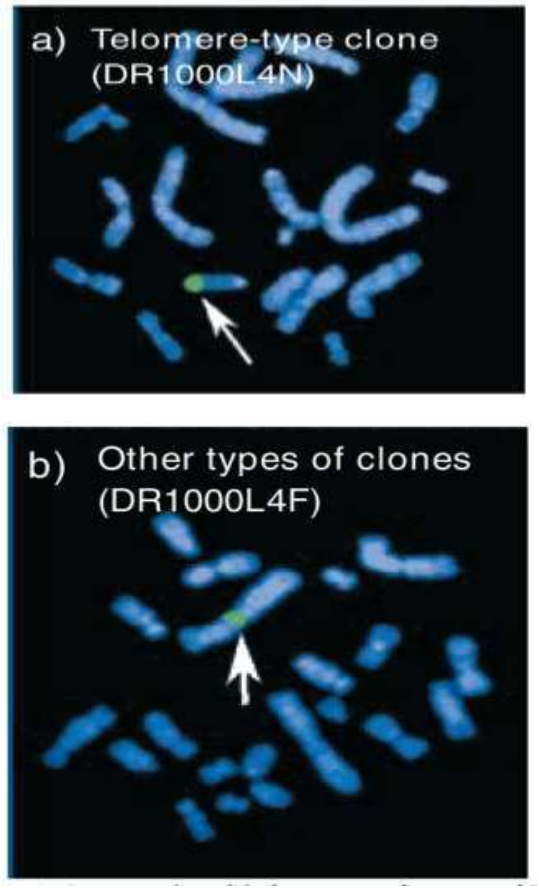
The DHFR activity (measured in IU) was estimated as follows:

$$\mathrm{IU} = \frac{\Delta A}{\epsilon \times d}\frac{V}{v} \times 10^3 \quad [\mathrm{units\ mL^{-1}}] \qquad (1)$$

where A is the absorbance-decreasing ratio [min-1], V is the total volume [mL], v is the crude enzyme solution volume [mL], and is the molecular extinction coefficient [12 000 M-1 cm-1] and d is the light path length [1 cm].

Results and Discussion:

We identified and characterized a variety of resistant cell clones to better understand how cell pools behave during stepwise selection. Using a limiting dilution for over a month, we were able to obtain a variety of clones that were resistant to 1000 nM of MTX. FISH was used to determine the location of the amplified gene after cloning. Figure 2 depicts the usual sites of inserted genes. The telomeric area (Figure 2a, DR1000L 4N) was home to several newly introduced genes, but other chromosomally dispersed genes were also found (Figure 2b, DR1000L-4F). They were known as telomeres and various forms of clones. Our short batch culture (4 days) allowed us to compare the properties of these clones, and we measured specific growth () and hGM-CSF production rates (F) during the logarithmic growth phase by FISH analysis (Table 2). Gene amplified cell pools, it was concluded from these findings, include a diverse range of geneamplified cells. To put it another way, different types of gene-amplified cells were induced during stepwise selection, resulting in heterogeneous geneamplified cell pools. The specific growth rate of half of the 26 clones was greater or equal to that of the original cell line, DG44. All of the high-specificproduction-rate clones were telomere-type clones, which was unexpected.

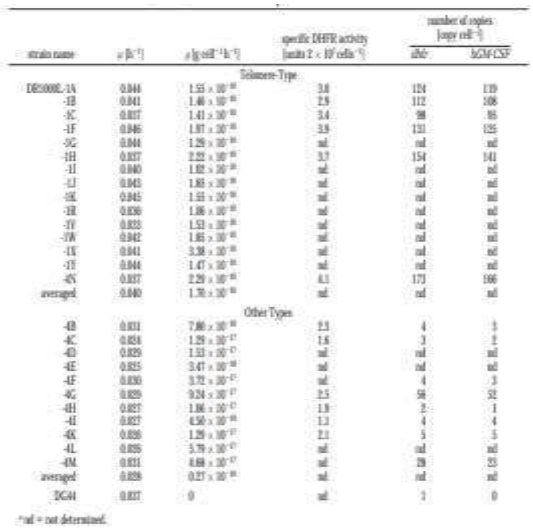


Amplified genes are shown in

Figure 2 to be located in chromosomal DNA by FISH. (a) Genes near the telomeric region that are amplified. There are genes on other chromosomes that have been amplified. The white arrow indicates the site of the amplified genes, and the count terstaining was performed using DAP

All other kinds of clones were outperformed by telomere-type clones in terms of particular growth rates. In comparison to other kinds of clones, the generation rate of hGM-CSF from telomere-type clones was roughly six times greater. A correlation was established between the productivity and growth rate of individual cell clones and the location of amplified genes, as determined by FISH analysis. Compared to heterogeneous cell pools, the growth and production rates of two kinds of clones in Table 2 were similar. A high percentage of telomere-type cells was found in heterogeneous cell pools when the quantity of MTX was increased over time. On the other hand, heterogeneous cell pools had fewer than 40% of telomere-type cells when MTX consumption grew rapidly. We used the dot-blot method to evaluate the specific DHFR activity and copy number of amplified genes, dhfr and hGM-CSF (Table 2), to better understand the distinctions between telomere-type and other kinds of clones.

Telomere-type clones did not vary significantly from other clones in terms of DHFR activity. A considerable difference was found between two kinds of clones in the study of copied genes. Telomere-type DNA was found in every single one of the samples.



compared to the total number of copies in the host cell line The dhfr and hGM-CSF genes are almost similar in their copy numbering. Telomere-type clones had around 100 times as many copies of both genes as the host cell line. There were much fewer copies of amplified genes in other genetically cloned creatures. The DR1000L-4G and DR1000L-4M clones have modest gene amplification, but not as much as the telo mere-type clones. They displayed robust growth and production rates, as well as a substantial copy number of the amplified gene (dhfr) and target gene (dhfr) (hGM-CSF). A number of species' telomeric regions depend on gene repression, also known as "gene silence," also known as "gene repression" (28). In order to endure the MTX stress, the cell must raise the expression of the dhfr gene if the number of dhfr copies at the telomeres drops. According to our study, we found that wild-type K1 and all telomere types had the same level of activity per copy of Dhfr. In normal Chinese hamster cells, the original dhfr gene was positioned in the "center" of chromosome 2q (29), far from the telomeric region. The telomeric region of the dhfr gene seems to have little effect on gene silencing. Figure 3b shows that the activity per copy of wild-type CHO K1 was almost similar to that of all telomere-type clones, with the exception of a few minor differences. As long as there are copies of dhfr in the telomeric region, gene silencing is improbable.

telomere-type genes may be expressed at lower levels than other kinds of genes for unknown reasons. To further comprehend these occurrences, more research will be needed, such as sequencing the amplified region and searching for clues.

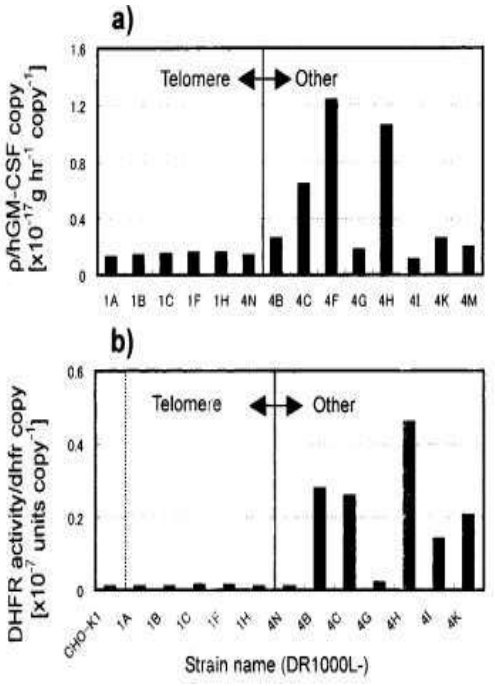


Figure 3 Two kinds of clones were studied for their specific production rates and DHFR activity per copy. HGM CSF copy production (a) and DHFR activity (b), on two clones, are shown in the top and bottom panels. The DR1000L-1 and L-4 cell pools were used to make these clones. There is a need for an enhancer/hot spot sequence at the telomeric region. We hypothesized that the variations in resistance mechanisms to MTX were to blame for the magnified level discrepancies. We computed the DHFR based on the DHFR activity data, the particular hGM-CSF production rate, and the number of copies of both genes.

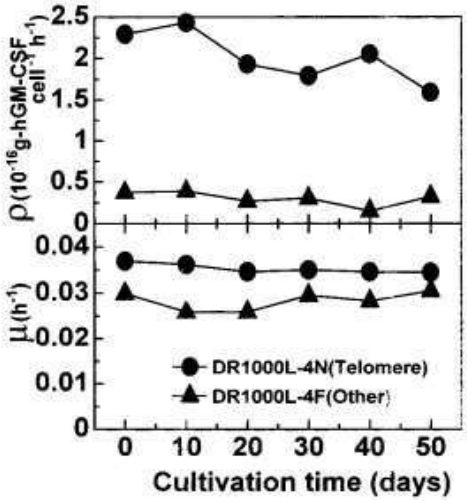


Figure 4 Long-term cultivation without MTX produces growth and production profiles. No MTX was employed in the growing process for 50 days. Growth and production rates were computed every 10 days. DR1000L-4N telomere-type clone (closed circles) and DR1000L-4F production rate (closed triangles) profiles are shown in Figure 3 for each DR1000L-4F hGM-CSF copy. When cells were repeatedly exposed to high concentrations of MTX, they developed a wide range of resistance. These cells may include both telomere- and non-telomeretype cells. It was postulated that telomere-type cells are more resistant to MTX than other kinds of cells. Telomere-type cells have lower DHFR activity per dhfr copy than most other cell types. As a result, gene amplification was critical in protecting the DHFR from the toxic effects of high doses of MTX. Most other cell types, on the other hand, showed considerable DHFR activity per dhfr protein copy. Amplification of the MTX gene was unnecessary. Amplification of MTX-resistant genes (9, 30) and membrane permeability alterations may not be necessary for cells to survive in the presence of the drug (31, 32). Thus, it's possible that MTX resistance has arisen in a variety of different clones in ways other than via gene amplification. There is evidence that SV40 and hCMV-MIE promoter sequence modifications have an impact on the specific production rate of recombinant IgG when used in the GS-NS0 system (33). Even with extremely low levels of dhfr gene expression, the DHFR-CHO system was able to generate substantial levels of gene amplification. An increase in particular productivity was achieved using gene amplification. Since recombinant proteins must be synthesized consistently, long-term culture without MTX has been utilized in industrial operations to make them. Figures 4 and 5 show stability and copy number testing of recombinant protein synthesis

utilizing telomere-type clones and other kinds of clones during a 50-day period without MTX. Over long periods of time, DR1000L 4N's growth and production rates did not alter much (Figure 4). Additionally,

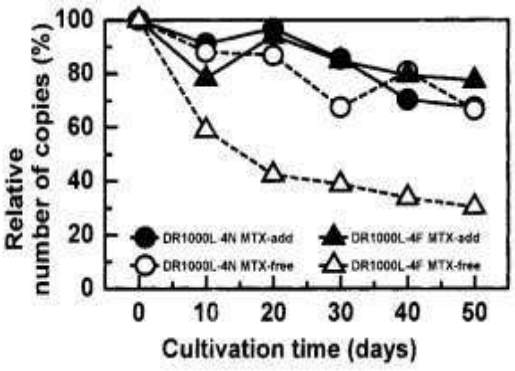


Figure 5. The number of copies of dhfr genes that have been amplified throughout time. Closed and open circles represent the presence or lack of MTX for the telomere-type clone, DR1000L-4N. Closed and open triangles show the presence or lack of MTX, respectively, for the DR1000L-4F clone. At the outset, there were 120 (DR1000L-4N) and 4 (DR1000L-4F) telomere-type clones of the dhfr gene (DR1000L-4F), respectively (Figure 5). For other clones that did not have MXX, the relative copy number for amplified genes reduced significantly. Finally, the telomere type clone was shown to be very stable in terms of productivity and gene amplification in the absence of MTX. Homologous recombination and translocations were common in CHO cells, making the karyotype unstable (34-36). In example, chromosomal rearrangements happened often during gene amplification. Cells that had been gene-amplified were always homogeneous. As a result, it's critical to know how diverse gene-amplified cell pools behave in order to fully grasp the process of gene amplification.

We used DR1000L-1, a heterogeneous geneamplified cell pool, to do long-term culture for 46 days with and without MTX, and then performed FISH analysis to determine the cell type distribution (14). In the absence of MTX, the percentage of telomere-type cells did not change during long-term culture, whereas the fraction of other cell types steadily reduced as the ratio of no signals increased. In the presence of MTX, the cell ratios of the two kinds of cells did not alter. Clone research revealed that telomere-type clones grew at a higher specific rate than other kinds of clones, according to the findings (Table 2). We concluded that telomere-type cells were able to survive and that the percentage of other kinds of cells reduced with time because these two types of clones were combined and cultured for a long period, which was comparable to the prior finding (14). Our finding was slightly corroborated by the results of the previous trial, which did not include MTX. This behavior was not found in heterogeneous cell pool culture when the mean specific growth rate of Telomere-type clones was roughly 1.4 times greater than that of other type clones (14). It was challenging to explain these longterm diverse cell pool behaviors merely in terms of the specific growth rate as assessed by clone analysis, based on earlier findings (14). It's likely that heterogeneous cell pools are the result of a combination of known and unknown variables, such as cell-cell contact and certain growth hormones. The heterogeneous cell pool as well as cloned cell properties must be analyzed to better understand the behavior of heterogeneous cells during gene amplification.

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