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

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ABSTRACT

In the past, in order to produce a genetically expanded CHO cell line that was not only extremely durable but also extremely prolific, we devised a procedure that could be carried out in a short amount of time, required little in the way of supervision, and was straightforward to carry out. This allowed us to generate a genetically expanded CHO cell line that was both exceptionally long-lasting and extremely prolific. Methotrexate (MTX) at higher concentrations led to the production of gene-amplified cell populations, which exhibited high and constant rates of specialized growth and output. These gene-amplified cell populations were shown to be resistant to the methotrexate. It would seem that the part of the chromosome's DNA that contained the bigger gene was responsible for the phenotypic characteristics that were shown by cells that had their genes amplified. We predicted that as we progressed through our exhaustive process of selection, a variety of unique gene-amplified cell types would eventually stand out to us as the most promising candidates. These clones were obtained from gene-amplified cell pools. This was done in order to get a deeper comprehension of how gene-amplified cell pools respond to a gradual rise in the amount of MTX present in the environmentThis led to the discovery of "telomere-type clones," which, in comparison to their conventional analogues, were more stable and produced a greater quantity of offspring. Following an analysis of the chromosomal DNA of telomere-type clones, it was found that these clones included more than one hundred copies of amplified genes. It is not unheard of to find less than 10 copies of amplified genes amongst a significant number of other clones; this is not an extremely rare occurrence. This is by no means something that happens just seldom. When, on the other hand, amplified genes were cultured for an extended period of time in other types of clones, MTX was not present; as a direct result, there was a rapid loss of the genes from the chromosomal DNA.

INTRODUCTION

The potential of genetic engineering is now more plausible as a result of this development. This process may be seen in the maturation of cancer cells as well as the development of treatment resistance in tumour cells and some human parasites. Other examples of this process include: (4, 5). In addition to that, it is possible that this procedure will result in the spread of cancer (see also 1, 2, and 3). (4, 5). These cells are used in the process of producing the drugs (6, 7). To the greatest degree possible, the mechanisms that are responsible for gene amplification have, up until this point in time, continued to be a mystery. It is now feasible to produce recombinant drugs in commercial settings by using a diverse variety of host cells, such as E. coli, yeast, and mammalian cells, amongst many others. This has made the process of manufacturing recombinant medications practical. Recombinant expression methods have been employed in commercial operations for the manufacture of granulocyte colony-stimulating factor (G-CSF), erythropoietin (EPO), and a number of other antibodies. These factors have been utilised in an attempt to stimulate the production of new colonocytes, and there has been some success (6, 7). This area has not yet reached its full potential, and there is still opportunity for advancement in the utilisation of mammalian cell lines in an industrial environment producing a product. This is another thing that has not been found yet. In addition, there was no consideration of alternative approaches for selecting candidates that were efficient, easy, and successful. Flow cytometry and cell affinity separation are only two examples of the types of processes that may be classified under this umbrella term. Gene amplification techniques that include the use of recombinant mammalian cells are often used. These techniques were developed as a means of compensating for the low level of productivity shown by mammalian cells while simultaneously allowing for the production of glycoproteins. These methods may be discovered in a wide variety of contemporary research facilities (6-8). One of these techniques for amplifying genes

is called the dihydrofolatereductase (dhfr) gene amplification method, and it was carried out in the ovaries of the Chinese hamster (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.

The bulk of the time, the cell line in question is: (8, 9). In order to apply gene amplification systems in industrial processes, it is essential to have selection procedures for recombinant CHO cell lines that can reliably yield the appropriate recombinant proteins (10-12). On the other hand, the techniques for selection have been carried out in an experimental manner so far, and their foundation is merely a process of trial and error. Throughout the course of long-term selection, we are interested in seeing how gene-amplified cell pools carry out their functions. In conclusion, one of our goals is to validate a strategy called quantitative selection in order to generate gene-amplified CHO cell lines that are both highly productive and stable over an extended period of time. Recent research on CHO cells has resulted in the development of a stepwise increase in MTX concentration.. Our investigation has led us to discover heterogeneous cell pools as a result of its findings. A number of genes that have been amplified are located in the telomeric region of chromosomal DNA. On the other hand, genes that were amplified were discovered in diverse cell pools that had lower rates of production and greater levels of instability. Throughout the process of selecting MTX-resistant cells in a step-by-step manner, there were some cells that were able to develop resistance while others were killed off. Cells that had altered membrane permeability and changes in the DHFR affinity for MTX survived the stepwise selection process. These cells include cells that were genetically amplified and cells that had modified the DHFR affinity. On the other hand, it is not yet understood how gene-amplified cell pools behave when they are subjected to selection. We extracted many various kinds of resistant cell clones and described the properties of these clones so that we could get a better understanding of how gene-amplified cell pools behave during stepwise selection. On the characteristics of the isolated clones, quantitative analysis and comparisons were carried out. These analysed characteristics included the rate of amplification, the copy number of amplified genes, and the stability of protein synthesis. 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. This discovery was made possible by the fact that the amplified genes were located near the telomeric region.

The Techniques and the Components the Cell Line, the Circumstances of the Culture, the Vectors, and the Execution of the Transfer

They were kept alive by providing them with IMDM (Sigma-Aldrich Japan, I-7633) with 10% FBS, hypoxanthine (13.6 mg L-1, Yamasa), and thymidine (2.42 mg L-1, Yamasa). IMDM was dialyzed with FBS at a concentration of 10%, but neither hypoxanthine nor thymidine was used in the selection process for dhfr+ (DG44-derived) transformants. It was stored in an atmosphere containing 5% CO2 and 37 degrees Celsius to ensure its integrity. The amount of MTX that was present in these cell pools was 1000 nM. The approach of controlled dilution was used to clone telomeres as well as for the purpose of this experiment, cell suspensions were diluted with IMDM medium to a concentration of one cell per 200 l before being plated out onto 96-well plates. Before being used in the research, cloned cells underwent expansion until they reached a cell density of 106 cells per millilitre of culture media. After that, some of them were culled, and the others were sold. This process took around a month to complete from start to finish. In order to make the pSV2-dhfr/hGM-CSF vector, the components ATCC 37146 and pcD-hGM-CSF were employed (17, 18). (Figure 1). The dhfr gene was introduced into three different vectors, including a pcD-hGM-CSF construct as well as two pSV2-dhfr variants. There were two different vectors, and both of them used SV40-polyA as a terminator for the promoters that they carried. Calculation of Specific Growth and hGM-CSF Production Rates with Instructions for Trans Fecation Broken Down Step-by-Step.

It was possible to stain just dead cells by diluting the cell samples to a volume of one using a solution containing 0.16 percent Trypan Blue and 0.85 percent NaCl. A Buerker-Turkhemacytometer was used in order to ascertain the total quantity of living cells (ERMA Tokyo, 4296). Omasa et al. went into an incredible amount of detail on this measurement process (20). The goal of this investigation is to evaluate the efficiency as well as the consistency of the built-in cell pools.

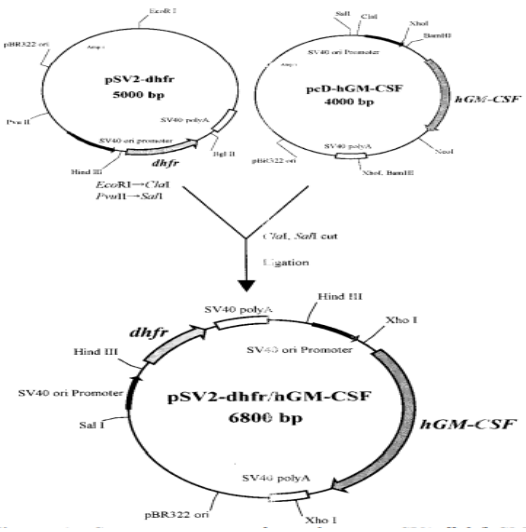


Figure 1. The steps that were required to be carried out in order to generate the pSV2-dhfr/hGM CSF vector were described here. The production of a pSV2-dhfr/hGM-CSF vector became possible when the pcD-hGM-CSF vector and the pSV2-dhfr vector were combined. After then, hGM-CSF may be manufactured with the use of this vector. The Dhfr gene and the hGM-CSF gene on this plasmid were organised in tandem such that the specific growth and human GM-CSF production rates (per cell per hour) could be calculated with a confidence level of 95 percent. In addition to that, the SV40 ori promoter as well as the SV40 polyA terminator were essential components. The data indicate that there is a degree of trust about the rates that is lower than 5 percent. This result was obtained via the use of an enzyme-linked immunosorbent assay (ELISA), which was performed on a plate with 96 individual wells (Greiner, 655061). Yoshikawa et al. presented a comprehensive analysis of the ELISA method, which included the estimation of specific growth and production rates (14).

Be Careful to Maintain a Record of the DHFR and hGM-CSF Statistics by Writing them Down and Monitoring them

Roche Diagnostics provided us with this specimen, which is documented with the accession number 1093657) During this specific step of the testing procedure, the accompanying instruction booklet for the DIG DNA labelling and detection kit was followed to the letter in order to guarantee correct findings. The use of restriction enzymes in the digesting process made it possible to produce two distinct kinds of probes (HinD III and Xho I, respectively). We were successful in recovering each fragment by employing an agarose gel electrophoresis separation technique to separate the digested fragments using a prepA gene DNA purification kit. This procedure included separating the fragments one at a time using a prepA gene DNA purification kit. Because of this, we were able to see each component on its own (Bio rad, 732-6010). There was a possibility of experimental error when carrying out the measurements of the dhfr gene copy number as well as when carrying out the measurements of the CSF gene copy number. The proportion of the whole that might be attributed to experimental error was much lower than ten percent. To determine how much chromosomal DNA was present, a fluorometry measuring method (22), in which Hoechst 33258 was utilised as the DNA-binding specific reagent, was used in order to collect the necessary data. This conclusion has been arrived at. In order to arrive at an average, this methodology was used. In contrast to RNA, this reagent has a very strong affinity for binding to the AT-rich region of this reagent. [Citation needed] (23). These two numbers are both expressed in nanometers (nm). The typical quantity of DNA found in a specimen was DNA. -DNA (Takara, 3010).

The Utilization of Fluorescence Hybridization in Clinical Circumstances (FISH)

For the production of chromosomal spreads, conventional techniques were used, and for this purpose, cells that were in the exponential phase of their development were utilised (24). The cells were allowed to remain in an environment that contained colcemid at a concentration of 10 grammes of colcemid per millilitre of media for a period of time ranging from 5 to 6 hours. The concentration of colcemid was maintained at 10 grammes of colcemid per millilitre of medium. The ambient was kept at a temperature of 37 degrees Celsius during the whole process. A solution containing 75 mMKCl was used to keep the cells alive at room temperature for a duration of twenty minutes while they were in the solution. The amount of solution that was used was 1.5 millilitres. After the hypotonic solution had been drained, a freshly crafted fixative with a MeOH to HOAc ratio of 3:1 was added to the mixture. The ratio of MeOH to HOAc was determined by the initial concentration of the combination. There was a three-step process that included depositing droplets of the suspension on a slide that was labelled Preclean and had been pre-cleaned with Matsunami (25, 26). During the course of this investigation, FISH was performed in a way that was in line with what Pinkel et al. described (27). The particulars of FISH have been covered in more length previously in the conversation (14). Because of the results, the amplified genes were able to be divided into three distinct categories, which made it feasible to carry out a computation about the proportion of amplified genes (telomere-type, other kinds, and those without signals).

Measurements are now Being Taken on the DHFR's Overall Activity Levels

One million cells were examined after their collection and subsequent resuspension in a total amount of PBS equal to 500 litres (PSB, pH 7.4). After being subjected to sonication for a period of five seconds and then being immersed in water that had been heated to a temperature of four degrees Celsius, the samples were analysed. In their separate roles, these procedures were carried out for a total of four distinct times during the course of their lifetimes. The crude enzyme was effectively isolated from the remainder of the mixture with the use of a five-minute centrifugation process at 18 000 g for five minutes. The concentration of a solution was determined by measuring the absorbance at 340 nm at a temperature of thirty degrees Celsius. The solution contained a 200 nM KH2PO4/KCl buffer, 500 L of 500 M dihydro folic acid (D7006), 290 L of ultrapure water, 10 mM a-NADPH, and 100 L of crude enzyme solution. The buffer had an initial concentration of 200 nM.

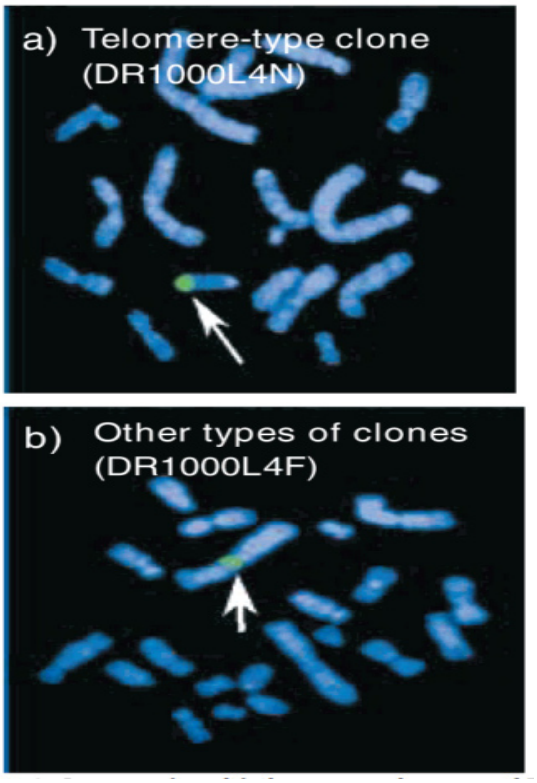
The following equation was used in order to arrive at a ballpark figure for the amount of DHFR activity (which was stated in IU):

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

where A represents the absorbance-decreasing ratio, which is expressed in units of min-1, V represents the total volume, which is expressed in millilitres, v represents the volume of the crude enzyme solution, which is expressed in millilitres, is the molecular extinction coefficient, which is expressed in units of 12 000 M-1 cm-1, and d represents the length of the light path, which is expressed in centimetres.

RESULTS

In order to have a better knowledge of the behaviour of cell pools during stepwise selection, we isolated and characterised a wide range of resistant cell clones. This allowed us to have a better handle on the situation. Because of this, we were able to have a greater comprehension of the procedure. Using a technique known as limited dilution over the course of more than a month, we were able to successfully produce a varied collection of clones that were resistant to the adverse effects of 1000 nM of MTX. This was possible because of the fact that we used such a long length of time. Following the conclusion of the cloning procedure, the FISH technique was used in order to locate the amplified gene in its exact location. Figure 2, which may be located by clicking this link, illustrates the typical placements of inserted genes. In addition to the newly inserted genes that were discovered in the telomeric region (Figure 2a, DR1000L 4N), other chromosomally scattered genes were also discovered in the sample (Figure 2b, DR1000L-4F). People at the time referred to them not only as telomeres but also as several other types of clones. Because of the short-batch culture that we used, which only lasted for a total of four days, it was possible for us to investigate the similarities and contrasts in characteristics that exist amongst these clones. During the logarithmic growth phase, FISH analysis was used in order to evaluate not only the specific growth rate but also the hGM-CSF production rate (Table 2). Because of these findings, the researchers came to the conclusion that gene-amplified cell pools include a wide variety of gene-amplified cells. The observations led to this conclusion, which was arrived at as a direct result of the observations. To put it another way, the process of stepwise selection resulted in the formation of heterogeneous gene-amplified cell pools, which were produced by the induction of various kinds of gene-amplified cells. These cell pools were produced as a result of the induction of gene-amplified cells. The progressive selection of gene-amplified cells yielded these cell pools as the product of that selection. The specific growth rate of half of the 26 clones was either larger than or equal to that of the parent cell line, DG44. This was the case for half of the clones. This was the circumstance with regard to sixteen of the clones. The rate of multiplication of each clone was measured, and using that data, a judgement was reached about the situation. The researchers were slightly taken aback when they made the startling finding that each and every one of the high-specific-production-rate clones was a telomere-type clone.



Amplified genes are shown in

Figure 2 Employing the FISH technique, in order to locate anything in the chromosomal DNA. (a) Amplification of genes that are the genome. There are genes that have been amplified, and they are present in the organism. These genes are located on different chromosomes, but they have been amplified. The counting was carried out with the help of DAP, and the direction of the arrow in white shows where in the genome the amplified genes are located.

When compared to the other kinds of clones that were investigated, the growth rates of the clones with telomeres were shown to be significantly superior to those of the other types. The rate at which telomere-type clones synthesised human gamma-macrophage colony-stimulating factor (hGM-CSF) was about six times higher than the rate at which other types of clones did so. Human gamma-macrophage colony-stimulating factor is what is meant by the abbreviation hGM-CSF. Because of the FISH analysis, it was possible to establish a connection between the levels of productivity and growth rate displayed by particular cell clones and the specific locations of amplified genes in their genomes. This connection was made possible by the fact that the FISH analysis was performed. It has been shown that there is a link between the two variables that are under investigation. The growth and production rates of the two different types of clones that are given in Table 2 were comparable to those of the heterogeneous cell pools when compared to those of the heterogeneous cell pools. This was the case when compared to those of the heterogeneous cell pools. When the amount of MTX was gradually raised over the course of a certain amount of time, it was discovered that heterogeneous cell pools had a

greater proportion of cells with telomeres. [Citation needed] DNA of the telomere type was discovered to be present in each and every one of the samples that were analysed.

strain name	μ [h^{-1}]	ρ [g cell^{-1} h^{-1}]	specific DHFR activity [units 2×10^7 cells^{-1}]	number of copies [copy cell^{-1}] dhfr	hGM-CSF
Telomere-Type					
DR1000L-1A	0.044	1.55×10^{-16}	3.0	124	119
-1B	0.041	1.46×10^{-16}	2.9	112	108
-1C	0.037	1.41×10^{-16}	3.4	98	95
-1F	0.046	1.97×10^{-16}	3.9	131	125
-1G	0.044	1.29×10^{-16}	nd	nd	nd
-1H	0.037	2.22×10^{-16}	3.7	154	141
-1I	0.040	1.02×10^{-16}	nd	nd	nd
-1J	0.043	1.85×10^{-16}	nd	nd	nd
-1K	0.045	1.55×10^{-16}	nd	nd	nd
-1R	0.036	1.06×10^{-16}	nd	nd	nd
-1V	0.033	1.53×10^{-16}	nd	nd	nd
-1W	0.042	1.85×10^{-16}	nd	nd	nd
-1X	0.041	3.38×10^{-16}	nd	nd	nd
-1Y	0.044	1.47×10^{-16}	nd	nd	nd
-4N	0.037	2.29×10^{-16}	4.1	173	166
averaged	0.040	1.70×10^{-16}	nd	nd	nd
Other Types					
-4B	0.031	7.80×10^{-18}	2.3	4	3
-4C	0.024	1.29×10^{-17}	1.6	3	2
-4D	0.029	1.53×10^{-17}	nd	nd	nd
-4E	0.025	3.47×10^{-18}	nd	nd	nd
-4F	0.030	3.72×10^{-17}	nd	4	3
-4G	0.029	9.24×10^{-17}	2.5	56	52
-4H	0.027	1.06×10^{-17}	1.9	2	1
-4I	0.027	4.50×10^{-18}	1.1	4	4
-4K	0.026	1.29×10^{-17}	2.1	5	5
-4L	0.026	5.79×10^{-17}	nd	nd	nd
-4M	0.031	4.68×10^{-17}	nd	28	23
averaged	0.028	0.27×10^{-16}	nd	nd	nd
DG44	0.037	0	nd	1	0

[a] nd = not determined.

when contrasted with the total number of copies that are present in the host cell line Within their respective genomes, the number of copies of the dhfr gene and the hGM-CSF gene is approximately identical. When the number of copies of both genes that were identified in the host cell line was compared to the number of copies that were found in telomere-type clones, the difference was almost one hundred times higher. The number of copies of amplified genes seen in other genetically cloned species, on the other hand, is far lower. Even though it is feasible to identify gene amplification in the DR1000L-4G and DR1000L-4M clones, the importance of this occurrence is not nearly as significant as it is in the telo mere-type clones. This is because the DR1000L-4G and DR1000L-4M clones are telo mere-type clones. These organisms demonstrated exceptional rates of growth and production, in addition to possessing a large copy number of both the amplified gene (dhfr) and the target gene (dhfr) (hGM-CSF). The process of gene repression, sometimes known as "gene silence" and on occasion referred to as "gene repression," is essential for the telomeric regions of a broad variety of different species. This is because gene repression prevents certain genes from being expressed (28). In the case that there is a decrease in the amount of dhfr copies that are situated at the telomeres, the cell will need to raise the expression of the dhfr gene in order to be able to withstand the effects of the MTX stress. According to the results of our research, we discovered that the amount of Dhfr activity per copy in wild-type K1 telomeres and in all other kinds of telomeres is the same. The first dhfr gene was found on chromosome 2q (29), a considerable distance from the telomeric region of the chromosome. The gene was found in the section of the chromosome that is known as the "core" of the chromosome. Normal Chinese hamster cells were used in the research that led to this result. It would seem that the telomeric region of the dhfr gene does not play a significant part in the process that results in the silence of genes. This is the conclusion that can be drawn from the available evidence. The information that is shown in Figure 3b demonstrates that the activity per copy of wild-type CHO K1 was almost exactly the same as that of all telomere-type clones, with just a few minute differences existing between the two groups of findings. It is exceedingly unlikely that genes will be silenced so long as the telomeric region contains several copies of the dhfr gene. This makes the possibility of gene silencing extremely remote. It is probable that, for reasons that are not entirely known, telomere-type genes are expressed at lower levels compared to other types of genes. The reasons for this are not completely understood. It will be necessary to do more study, such as sequencing the amplified area and looking for ideas, in order to get a more in-depth understanding of these events. This will allow for a more complete comprehension of the situation.

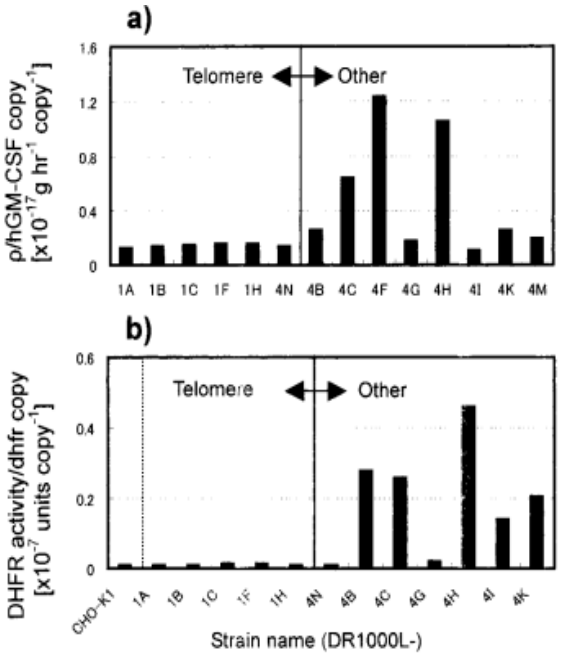


Figure 3The particular production rates of both types of clones, as well as the DHFR activity per copy, were investigated as part of this experiment. The HGM CSF copy production (a) and DHFR activity (b) are shown, separately, on clones that are unique from one another in the top and bottom panels, respectively. When these clones were originally formed, they were first created from the DR1000L-1 and L-4 cell pools, respectively, and then these clones were initially separated from those cell pools. In order for it to carry out its intended purpose, the telomeric region absolutely has to have either an enhancer or a hot spot sequence embedded inside it. This was our working hypothesis, and it said that the amplified level variations were due to changes in the resistance mechanisms to MTX. Specifically, we thought that these changes were the cause of the differences. This was the statement that was included in a hypothesis that we had, and it read as follows: The data on DHFR activity, the specific hGM-CSF production rate, and the number of copies of both genes were used in our calculation of the DHFR. This allowed us to determine the DHFR. Because of this, we were able to compute the DHFR.

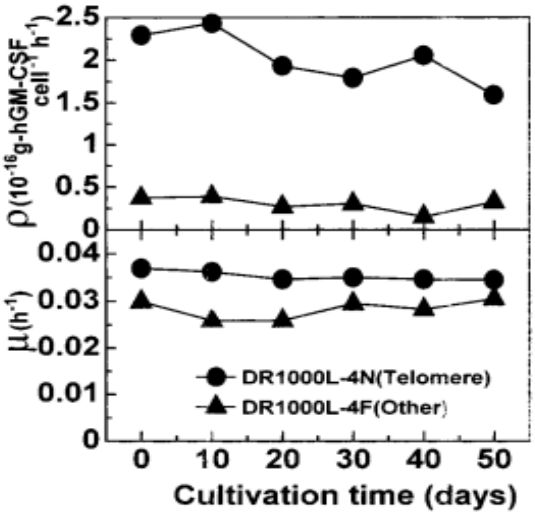


Figure 4 The absence of MTX throughout the cultivation process for an extended period of time may lead to the formation of growth and production profiles. For the duration of the growth process, there was a period of fifty days in which MTX was not used in any way, shape, or form. A computation was carried out once every ten days in order to calculate the growth and output rates. Figure 3 depicts the DR1000L-4N telomere-type clone profiles as closed circles, and Figure 3 depicts the DR1000L-4F production rate profiles as closed triangles for each DR1000L-4F hGM-CSF copy. Both sets of profiles are given for each DR1000L-4F hGM-CSF copy. When cells were repeatedly exposed to high concentrations of MTX, they started to evolve a broad array of defence mechanisms that enabled them to tolerate the effects of the toxin. These mechanisms allowed the cells to survive prolonged exposure to the toxin. It is not completely out of the question for these cells to have components that are not of the telomere type in addition to those that do contain telomere-type components. It is hypothesised that telomere-type cells, in comparison to other types of cells, have a greater capacity to withstand the effects of MTX. [Citation needed] [Citation needed] Telomeres are a kind of genetic material, which explains why this is the case. When compared to the overwhelming majority of other cell types, telomere-type cells stand out from the crowd due to the fact that they have unusually low levels of DHFR activity per dhfr copy. Amplification of genes was thus an essential step in the process of shielding the DHFR from the potentially lethal effects of large doses of MTX. This was accomplished via the process of gene amplification. The fact that gene amplification was a consequence of this situation is what brought about this finding. On the other hand, the vast majority of other cell types had a considerable level of DHFR activity in comparison to the amount of dhfr protein they possessed. This was the case regardless of the kind of cell. The existence of the MTX gene was not required for the amplification of the MTX gene to take place. It is likely that the amplification of MTX-resistant genes (9, 30) and variations in membrane permeability are not required for cells to be able to survive in the presence of the medication. [Cells can survive in the presence of the medication because] [Cells can survive in the presence of the medication because] [Cells can survive in the This is something that need further research and investigation (31, 32). Therefore, it is plausible that MTX resistance emerged in a number of distinct clones as a consequence of processes other than the amplification of genes in each of their individual genomes. This is something that needs more investigation. This is a plausible explanation for the phenomenon. When the GS-NS0 system is used, there is evidence to suggest that alterations to the sequence of the SV40 and hCMV-MIE promoters have an effect on the specific production rate of recombinant IgG. This is the situation due to the fact that the promoters for SV40 as well as hCMV-MIE are situated in the same region of the gene (33). Even when the levels of dhfr gene expression were rather low, the DHFR-CHO system was still able to create a large amount of gene amplification. This was due to the system's ability to produce high levels of homologous recombination. As a direct consequence of gene amplification, there was the potential for a substantial increase in the overall quantity of a given component that might be generated. When recombinant proteins are produced in an industrial environment, it is common practise to use the utilisation of long-term culture that does not necessitate the application of MTX. This is due to the fact that the production of recombinant proteins requires that the creation of these proteins be done in a manner that is consistent. Figures 4 and 5 demonstrate the results of stability and copy number testing conducted on recombinant protein synthesis using telomere-type clones and other types of clones, respectively, for a period of fifty days during which there was no MTX present. This testing was conducted. The tests was performed as described. There was not a detectable change in the growth or production rates of the DR1000L 4N throughout the course of extended periods of time (Figure 4). Additionally,

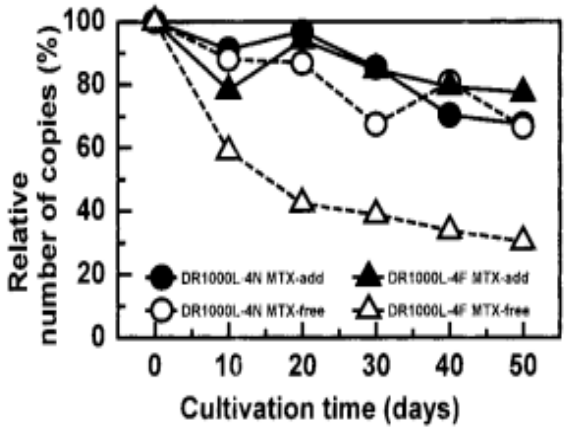


Figure 5. The total number of dhfr gene copies that have been amplified as a result of the progression of human history. The telomere-type clone DR1000L-4N either has MTX or it does not; this may be determined by looking at the closed or open circles, respectively. In the case of the DR1000L-4F clone, the presence of MTX is signified by a triangle with its three points closed, while the lack of MTX is shown by a

triangle with its three points open. There were a total of 120 telomere-type clones of the dhfr gene (DR1000L-4N) and a total of 4 telomere-type clones of the dhfr gene (DR1000L-4F) when the experiment first began (Figure 5). When compared to the copy number of amplified genes discovered in other clones that did not include the MXX gene, the relative copy number was much lower. In conclusion, it was shown that the telomere type clone was generally stable with regard to productivity and gene amplification even when MTX was absent from the surrounding environment. CHO cells were notorious for having an unstable karyotype due to the high rate of homologous recombination and translocations that took place in these cells. As a consequence, the karyotype of CHO cells became infamous (34-36). A good illustration of this is the fact that the process of amplifying genes resulted in a number of different chromosomal rearrangements. The creation of cell populations that are genetically similar to one another is an inevitable consequence of the process of gene amplification. Therefore, in order to have a complete understanding of the process of gene amplification, it is essential to be familiar with the behaviours of various gene-amplified cell pools. This is because different gene-amplified cell pools behave in different ways. This is due to the fact that the synthesis of numerous copies of a gene is an integral part of the process of gene amplification.

Tests on long-term culture were carried out using the heterogeneous gene-amplified cell pool DR1000L-1 for a period of 46 days. These experiments were carried out with and without the addition of MTX. After that, we performed FISH analysis in order to assess the relative abundance of the different kinds of cells that were found in the sample (14). In the long-term culture without MTX, the proportion of cells that had telomeres did not change; nevertheless, the percentage of other cell types steadily decreased as the ratio of cells that had no signals grew. This was in stark contrast to the situation that had existed throughout the time when MTX was there. The cell-to-cell ratios of the two different types of cells did not seem to experience any noticeable changes when MTX was present. This was the case even when the ratios were being measured in the presence of MTX. The outcomes of the research demonstrated that the rate of growth of telomere-type clones was much faster than that of the growth rate of other types of clones. Research on clones led to the discovery of this fact (Table 2). We came to the conclusion that telomere-type cells had the ability to survive and that the percentage of other types of cells decreased over time as a result of combining these two different types of clones and cultivating them for an extended period of time. This was the result of combining these two types of clones and cultivating them for an extended period of time. The finding that was reported in the prior research was comparable to the one that was found in this investigation. In addition, we discovered that the proportion of cells belonging to other kinds fell during the course of the study (14). Our conclusion was given some credibility by the findings of the earlier experiment, which did not include the use of MTX, and we were able to verify our hypothesis with their assistance. This trend was not seen in cultures consisting of diverse cell pools, in which the average specific growth rate of telomere-type clones was about 1.4 times higher than that of other types of clones (14). According to the results from past studies, it was difficult to provide an explanation for these long-term behaviours of various cell pools using just the particular growth rate as determined by clone analysis (14). There is a good chance that heterogeneous cell pools are the consequence of the interaction of a number of different factors, some of which are known and others of which are unknown. This is a notion that deserves substantial consideration. Cell-to-cell interaction and the production of specific growth hormones are two examples of these variables. It is necessary to perform an analysis on the heterogeneous cell pool as well as the characteristics of cloned cells in order to get a better understanding of the behaviour of heterogeneous cells when genes are being amplified. This will allow for a better comprehension of the way in which heterogeneous cells behave when genes are being amplified.

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