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The isolation of CHO cells with a site conferring a high and reproducible transgene amplification rate

Jonathan J. Cacciatore[a], Edward F. Leonard[a], Lawrence A. Chasin[b,*]

[a] Department of Chemical Engineering, Columbia University, New York, NY 10027, United States
[b] Department of Biological Sciences, Columbia University, New York, NY 10027, United States

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ABSTRACT

Co-amplification of transgenes using the dihydrofolate reductase/methotrexate (DHFR/MTX) system is a widely used method for the isolation of Chinese hamster ovary (CHO) cell lines that secrete high levels of recombinant proteins. A bottleneck in this process is the stepwise selection for MTX resistant populations; which can be slow, tedious and erratic. We sought to speed up and regularize this process by isolating *dhfr*$^-$ CHO cell lines capable of integrating a transgene of interest into a defined chromosomal location that supports a high rate of gene amplification. We isolated 100 independent transfectants carrying a gene for human adenosine deaminase (*ada*) linked to a φC31 attP site and a portion of the dihydrofolate reductase (*dhfr*) gene. Measurement of the *ada* amplification rate in each transfectant using Luria–Delbruck fluctuation analysis revealed a wide clonal variation; sub-cloning showed these rates to be heritable. Site directed recombination was used to insert a transgene carrying a reporter gene for secreted embryonic alkaline phosphatase (SEAP) as well as the remainder of the *dhfr* gene into the attP site at this location in several of these clones. Subsequent selection for gene amplification of the reconstructed *dhfr* gene in a high *ada* amplification candidate clone (DG44-HA-4) yielded reproducible rates of *seap* gene amplification and concomitant increased levels of SEAP secretion. In contrast, random integrations of the *dhfr* gene into clone HA-4 did not yield these high levels of amplification. This cell line as well as this method of screening for high amplification rates may prove helpful for the reliable amplification of recombinant genes for therapeutically or diagnostically useful proteins.

1. Introduction

The phenomenon of gene amplification is often exploited to produce therapeutic recombinant proteins, monoclonal antibodies being the prime example. A gene or genes coding for the therapeutic protein(s) of choice (e.g., monoclonal antibody heavy and light chain) are transfected into a host cell along with a selectable drug-resistance marker gene. After an initial isolation of transfectants exhibiting a minimal level of drug resistance, selection for transfectants that have acquired a modestly higher level of resistance by virtue of amplification of the marker gene is carried out. Many iterations of this selection process can result in cell clones with a high level of resistance, a high level of marker gene expression, and a high number of copies of the marker gene. Since the size of the amplified region is much larger than a single gene (Hamlin, 1992) and since transfected genes usually co-integrate in the host genome, even when co-transfected on separate plasmids (Chen and Chasin, 1998), the gene of interest is also co-amplified and its protein produced at a high level (Wigler et al., 1980).

A popular system for this approach uses a gene specifying the enzyme dihydrofolate reductase (DHFR) as the selective marker (Ringold et al., 1981), methotrexate (MTX) as the drug and a Chinese hamster ovary (CHO) cell line deficient in this ubiquitous enzyme (Urlaub and Chasin, 1980; Urlaub et al., 1983; Jayapal et al., 2007) as a host. CHO cells are capable of gene amplification, a trait not shared by normal cells (Livingstone et al., 1992; Wahl et al., 1984a; Yin et al., 1992). In this case the target of the drug is DHFR, and resistance is often gained by overproducing it. Transfected cells are cultured in a medium lacking a source of purine and thymidine nucleotides. Since DHFR-deficient host cells are unable to synthesize these metabolites only the transfectants can grow. MTX is a specific and tight-binding inhibitor of DHFR, but amplified cells that have undergone *dhfr* amplification ("amplificants") can overcome a judiciously chosen concentration of the drug.

Although the *dhfr*/MTX gene amplification method can result in as much as a 1000-fold increase in gene copy number (Kaufman and

Abbreviations: CHO, Chinese hamster ovary; DHFR, dihydrofolate reductase; MTX, methotrexate; ADA, adenosine deaminase; dCF, deoxycoformycin; SEAP, secreted embryonic alkaline phosphatase; PTF, purine and thymidine free.

* Corresponding author at: 912 Fairchild Center, Mail Code 2433, Columbia University, New York, NY 10027, United States. Tel.: +1 212 854 4645; fax: +1 212 531 0425.
E-mail address: lac2@columbia.edu (L.A. Chasin).

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Sharp, 1982), it suffers from long and variable development times. Each amplification step brings about only a modest increase in gene copy number, thus severely limiting the concentration of MTX that can be applied at each step. It is common to take six months or longer to isolate a cell line with the desired recombinant protein production level. This time bottleneck inhibits the rapid testing of multiple new candidates for pre-clinical evaluation and ultimately limits how quickly a new drug candidate gets to market (Trill et al., 1995). Use of MTX amplification may also have unforeseen effects on CHO cells since chromosomal translocations often accompany gene amplification. Additionally, amplified genes are not always stable, resulting in a decrease in gene copy number unless selective pressure is maintained. Due to these drawbacks, this approach is often not used despite its potential to yield high gene copy number clones. Industry has reacted by using novel promoter-enhancer and chromatin opening elements and developing high-throughput techniques to isolate rare high producing clones. Despite these drawbacks, regularizing this process and decreasing the time to implement it could lead to its more widespread use in the development of useful biologics.

Although the factors influencing gene amplification are not well understood, it has been shown that clones within a CHO transfectant population exhibit a wide distribution in the yield of amplificants (Kito et al., 2002; Wahl et al., 1984b). The simplest explanation for this variability is a position effect within the CHO genome. We reasoned that the gene amplification regimen could be both shortened and made more reliable by inserting the gene of interest along with the *dhfr* gene at a defined locus in the CHO genome chosen for supporting a high amplification rate. We identified such sites by screening 100 transfectants for their rate of amplification (as opposed to the frequency of amplified cells) using Luria–Delbruck fluctuation analysis. A chosen site could subsequently be used to insert genes of interest via an included site specific recombination sequence. A winning clone was shown to amplify a *dhfr* gene along with a gene of interest at a rate higher than randomly integrated transfectants and to secrete a protein of interest at increased levels. This clone or similarly isolated clones may prove advantageous for those using gene amplification in CHO cells to drive high level production of therapeutically or diagnostically useful proteins.

2. Materials and methods

2.1. Plasmid construction

We constructed a vector (pKAPD1) to be transfected into host DHFR-deficient DG44 cells (Urlaub et al., 1983, 1986) carrying a neomycin resistance gene and an amplifiable human adenosine deaminase (*ada*) gene. A portion of the *dhfr* gene and a φC31 attP site were included for subsequent recombination and amplification steps. A fragment containing the *dhfr* promoter, exon 1 and part of intron 1 was isolated by PCR using primers appended with AscI restriction site ends (forward: ACGTAGGCGCGCCGGGGCCTCTGATGTTCAAT; reverse: ACGTAGGCGCGCCCCCCTCATGACTGTCCCTAA), digested with AscI, and ligated into an AscI site that had been inserted into a unique BglII site on plasmid pEGFP-C3 (ClonTech) so as to retain one BglII site. A synthetic 39 bp attP site for φC31 recombinase (Thyagarajan et al., 2001) was then ligated into the unique BglII site downstream of the *dhfr* insert resulting in plasmid pKPD1. A human adenosine deaminase gene (*ada*) driven by a CMV promoter was isolated by ApaLI digestion from plasmid pCMV*ada* (American Type Culture Collection). The insert was ligated into the unique ApaLI site in pKPD1 to create vector pKAPD1 containing the *dhfr* promoter and exon 1, the attP site, a kanamycin/neomycin resistance gene, and the *ada* gene in that order (see top of Fig. 2). Next, we constructed a plasmid (pBE26) with an attB site to be used for recombination into the aforementioned attP site. PBE26 also contains the remaining 3′ portion of the *dhfr* gene and a *seap* gene coding for secreted embryonic alkaline phosphatase (SEAP) for future protein assays. A plasmid was first created by removal of the *dhfr* promoter, exon 1 and part of intron 1 from pDCH1P11 by digestion at a unique SmaI site upstream of the promoter and a unique PstI site within intron 1. The plasmid pDCH1P11 was previously constructed (Noe et al., 1999) by cloning a *dhfr* gene, driven by the *dhfr* promoter and containing only intron 1 and one natural polyadenylation signal, between unique SmaI and HindIII restriction sites in pSP72 (Promega). A synthetic 34 bp φC31 recombinase attB site (Thyagarajan et al., 2001) was ligated into a unique PstI site in intron 1 upstream of the contiguous *dhfr* exons 2 through 6. To create a vector directing the synthesis of SEAP (pSEAPBE26, Fig. 2), a region of pBE26 comprised of the attB site, *dhfr* exons 2 through 6 and a *dhfr* polyadenylation site was isolated by PCR using primers with NotI restriction site ends (forward: ACGTAGCGGCCGCGCCGATTCATTAATGCAGGT; reverse: ACGTAGCGGCCGCTGCTCTCAGGGGCTCTATGT) and ligated into the unique NotI site in the plasmid pCMVSEAP (Addgene #24595) downstream of the *seap* gene driven by a CMV promoter.

2.2. Stable transfection

Vector pKAPD1 was linearized by digestion at a unique AgeI restriction site and transfected into host CHO DG44 cells by electroporation with a low DNA concentration (20 ng/10^7 cells/ml) to promote single copy integration. Stable transfectants were selected with 800 μg/ml G418 for 2–3 weeks with medium renewals about every five days. One hundred colonies were isolated with cloning cylinders, expanded and frozen.

2.3. Fluctuation tests

Fluctuation analysis was used to measure the rate of amplification of the adenosine deaminase (*ada*) gene. High levels of this enzyme afford resistance to a combination of high adenosine and deoxycoformycin (dCF), an ADA inhibitor, and dCF resistance in *ada* transfectants arises by amplification of the transgene (Kaufman et al., 1986). A pilot study performed with several *ada* transfectant clones showed that the inclusion of 0.5 μM dCF reduced survival in the presence of 1.1 mM adenosine to an average of about 10 colonies per 10^6 treated cells. Each of the 100 G418-resistant stable clones was thawed, expanded, and seeded into 12 wells of a 96-well dish at a density of approximately 10 cells per well so that inclusion of a preexisting amplificant was highly unlikely. Cells in each well were grown to confluence and then expanded into 12 wells in 6-well dishes. At about 75% confluence the cells in each well were challenged with 0.5 μM dCF plus 1.1 mM adenosine for 2–3 weeks. Surviving colonies were fixed with 3.7% formaldehyde and stained with crystal violet. Colony counts from the 12 wells were used to calculate an amplification rate (events/cell/generation) using the P_0 and mean methods (Luria and Delbruck, 1943) and the median method (Lea and Coulson, 1949). A given method did not always allow a calculation of an amplification rate; in these cases we were still able to calculate a maximum or minimum rate, as follows. Sometimes all 12 cultures yielded dCF-resistant colonies, undermining the calculation of P_0, the proportion of cultures yielding no colonies. In these cases we estimated a minimum amplification rate by assuming that the thirteenth culture would have yielded no colonies, i.e., a P_0 of 1/13. In the opposite situation, when none of the 12 cultures yielded resistant colonies we assumed the 13th culture would have yielded colonies, for a P_0 of 12/13, so that the amplification rate was a maximum estimate. In other cases some wells contained colonies too numerous to count; here we assigned