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Amplification and expression of recombinant genes in serum-independent Chinese hamster ovary cells

Christine Gandor[a,**], Christian Leist[a,**], Armin Fiechter[a], Fred A.M. Asselbergs[b,*]

[a]*Department of Biotechnology, Swiss Federal Institute of Technology, CH-8093 Zürich, Switzerland*
[b]*Pharma Research Department, CIBA-GEIGY, CH-4002 Basel, Switzerland*

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Abstract CHO SSF3 cells grow as a suspension culture in unmodified commercial medium with only low-molecular weight ingredients. Continuous serum-free culture unexpectedly induced expression of a low dihydrofolate reductase activity in the originally *dhfr*⁻ CHO cells. Nevertheless, it was possible with methotrexate to induce amplification of a gene coding for the hybrid plasminogen activator K2tu-PA cotransfected with a *dhfr* gene. Expression of K2tu-PA expression was proportionally increased to that of *dhfr*, which was measured with fluorescent methotrexate. Because no serum proteases were present, secreted K2tu-PA was not converted to the enzymatically active form, but was exclusively recovered in proenzyme form.

Key words: CHO cell; DHFR; Serum-free; Gene amplification; Plasminogen activator

1. Introduction

Chinese hamster ovary cells lacking the enzyme dihydrofolate reductase, CHO(dhfr⁻), are among the most frequently used recipient cells for stable transfection experiments. Cell lines, in which the recombinant *dhfr* gene together with cotransfected genes is amplified, are the basis of a very successful method to produce (glyco-)proteins in mammalian cells, which are then used for research purposes or as pharmaceutical products [1–2]. The presence of serum in the conventional culture media complicates biological assay systems and is a negative determinant of the cost and quality of recombinant proteins [3,4]. Also ethical considerations press the scientific and industrial community to find ways to limit the slaughter of bovine fetuses for their serum. Hence, it is a great advantage to have cell lines which proliferate without proteinaceous growth factors. The development of such cell lines requires both optimization of the composition of the low-molecular weight segment of the culture medium [5] and development of a cell line adapted to grow in this medium without addition of macromolecular growth factors [6].

Here, we describe the isolation of CHO cell variants adapted to grow in a commonly used, commercially available protein-free medium. As an example of its use, the production of the hybrid plasminogen activator K2tu-PA is given. This protein is currently investigated in a clinical trial as treatment for acute myocardial infarction. It is composed of the Kringle-2 domain of human tissue-type plasminogen activator (t-PA) attached to the serine protease domain of the human urokinase-type plasminogen activator (u-PA) [7–11]. Like in the native plasminogen activators, proteolytic cleavage converts the otherwise inactive proenzyme K2tu-PA into an active plasminogen activator. Unless protease inhibitors are added, this activation of plasminogen activators occurs spontaneously in serum-containing cell cultures. The article focuses specifically on expression of *dhfr*, the spontaneous reactivation of a silent *dhfr* gene in the serum-independent cell variants and on the amplification of *dhfr*-linked genes in the new cell lines.

2. Materials and methods

2.1. Cell culture and DHFR assay

Serum-dependent CHO(dhfr⁻) cells of strain DXB11, also referred to as DUKX, DUKXB1 or DUK-XB11 (a gift of Dr. L. Chasin), and CHO-K1 (ATCC CCL-61) were cultured with 4% fetal calf serum as previously described [11]. Lipofection of SSF3 cells with *dhfr* and K2tu-PA genes was done in DMEM/F12 medium with 4% FCS as described previously [11,12]. Initial selection of transformants was done in media lacking glycine, hypoxantine and thymidine supplemented with 5 nM methotrexate (Sigma) and 4% dialyzed FCS. Selection for clones with increased *dhfr* expression was done by gradually increasing the methotrexate concentration [11,12]. Cellular DHFR enzyme activity was assayed spectrophotometrically [13] or by FACS analysis of cells labeled with fluorescent methotrexate as described previously [14].

2.2. Protein analysis

The amount of K2tu-PA in conditioned media was measured using an ELISA procedure with two monoclonal antibodies recognizing the Kringle-2 domain of tissue-type plasminogen activator in the hybrid protein [7]. The cell number was determined using the hexoseaminidase method [15]. K2tu-PA was purified by immuno-affinity chromatography using monoclonal antibody 405-B-33-3 [7]. The percentage single-chain K2tu-PA was analysed by polyacrylamide gel electrophoresis of the purified material.

3. Results

3.1. Choice of the culture medium

The CHO(dhfr⁻) mutant DXB11 used in our experiments is derived from the proline-auxotrophic cell line CHO-K1 [16,17]. Hence, a proline-containing medium had to be chosen with additional supplements to compensate for the lack of the DHFR enzyme in the cells. DHFR catalyzes the reduction of folic acid to dihydrofolic acid and hence to tetrahydrofolic acid. In the absence of folate in the medium or in the absence of intracellular DHFR activity, cells exhibit a requirement for glycine, a purine and thymidine. The purine requirement is usually satisfied with hypoxanthine or adenine. In our laboratory, DXB11 are grown in alpha-MEM medium supplemented

*Corresponding author. Pharma Research Department, CIBA-GEIGY K681.4.42, CH-4002 Basel, Switzerland.
Fax: (41) (61) 6969301. E-mail: asselber@FMI.ch

**Present address:* Pharma Research Department, CIBA-GEIGY, CH-4002 Basel, Switzerland.

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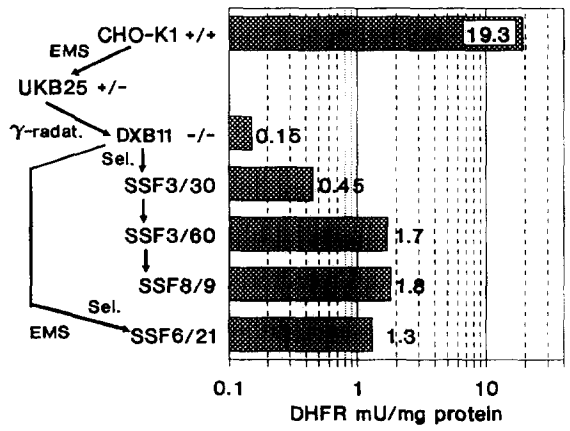

Fig. 1. DHFR expression in different CHO cell lines. The dhfr$^-$ CHO cell line DXB11 was isolated after mutagenesis first with ethyl methane sulfonate (EMS), creating the intermediate hemizygous UKB25 line followed by treatment with gamma radiation [16,21]. From DXB11, the different SSF lines were derived with or without EMS mutagenesis by selection (Sel.) for growth in protein-free medium. SSF3 cultures were tested for DHFR activity as described in section 2 after being cultured for 30 or 60 passages in protein-free medium. At passage 60, a SSF3 subline termed SSF8 was isolated, which was tested 9 passages later.

with ribo- and deoxyribonucleosides (Life Sciences) and 4% FCS. The alpha-MEM medium is very rich in essential and non-essential amino acids and contains 40 mg/l proline and 50 mg/l glycine. The glycine plus the added nucleosides allow *dhfr*$^-$ cells to grow. The *dhfr*$^-$ phenotype of DXB11 appears to be stable over at least 20 passages in this medium as judged from the lack of 'background colonies' in *dhfr* transfection experiments. It was not possible to isolate clones of CHO(dhfr$^-$) cells, which would grow in serum-free alpha-MEM. Hence, DMEM/F12 medium was used instead. It contains compared with alpha-MEM many additional components. The glycine, hypoxanthine and thymidine content are 18.75 mg/l, 2.39 mg/l and 0.365 mg/l, respectively. Hence, DMEM/F12 is expected to be non-selective with respect to the *dhfr*-status of the cells.

3.2. Isolation and cultivation of serum-independent CHO cells

Isolation of cell lines, which grow in DMEM/F12 medium completely devoid of macromolecular components, is schematically shown in Fig. 1. One series of cell lines, of which SSF6 is typical, was obtained by ethyl methanesulfonate (EMS) mutagenesis of DXB11 cells followed by selection in protein-free DMEM/F12, whereby 30% of the medium was replaced 2–3 × per week. A second series of cell lines, including SSF3 and its derivatives, were obtained by the same method, but without prior EMS mutagenesis. Using either method, it took 6–8 weeks before extensive proliferation of the cells could be detected. Both the SSF3 line and the SSF6 line have since been cultured for more than a year under protein-free conditions. Precisely, because it was never subjected to deliberate mutagenesis, most of our gene expression experiments have been done with the SSF3 line.

The protein-independent phenotype of the SSF cells appears to be stable. SSF3 cells grown for 4 months in DMEM/F12 with 4% serum could be re-adapted to protein-free medium without any apparent lag in cell growth rate. When standard CHO cells (*dhfr*$^-$ or *dhfr*$^+$) were similarly placed in serum-free medium, after 3–4 days the cells gradually started to die to the extent that < 10% of the initial cells were viable after a week. The proliferation rate of SSF cells in DMEM/F12 is close to that of the parental DXB11 cells in medium with serum (specific growth rate, $\mu = 1.1$ d^{-1} [18,19]). The actual specific growth rate of SSF3 ranged from 0.38 d^{-1} at low density to 0.9 d^{-1} at high cell concentrations. This dependence of the proliferation rate on the density at which the cells are seeded was lost when 30% conditioned medium of SSF3 cells was added to the medium. Stimulation of the growth of DXB11 cells in SSF cell-conditioned medium also suggest that the SSF cells produce (dialyzable) growth-promoting substances [18].

3.3. SSF cells grow as suspension cultures

When grown in protein-free medium, SSF cells did not adhere stably to the plastic of standard tissue culture dishes. After being subcultured in fresh medium, the cells attached transiently, but did not spread out. However, 2 or 3 days after subculturing all the SSF cells detached from the plastic and proliferated even in the absence of stirring as a suspension culture. This detachment from the growth substrate may explain why serum-independent CHO cells have not been isolated much earlier. After addition of serum, SSF cells attached rapidly to the growth substrate and, once spread out, were in the light microscope morphologically indistinguishable from the parental CHO(dhfr$^-$) cells. Therefore, the 'non-adherent-growth' phenotype of the SSF cells is probably not the direct result of a genetic change of the cells. Apparently, the SSF cells and presumably also the parental CHO cells do not make sufficient adherence proteins to adhere to the growth substrate in absence of factors normally added with the serum. That the SSF cells do not attach to a growth substrate in absence of serum proteins, is an advantage in large scale cultures, as the SSF cells do not cling to the interior surface of bioreactors, nor do they obstruct the surface of in-line detection devices.

3.4. Reactivation of a silent endogenous dhfr gene in SSF cells

Although, SSF cells have never been exposed deliberately to *dhfr*-selective conditions, we discovered that most of the cell lines, especially those cultured longer under protein-free condition, continued to grow when transferred to selection medium (Fig. 2). The growth difference in selective and non-selective medium disappeared when cells were plated at high cell density.

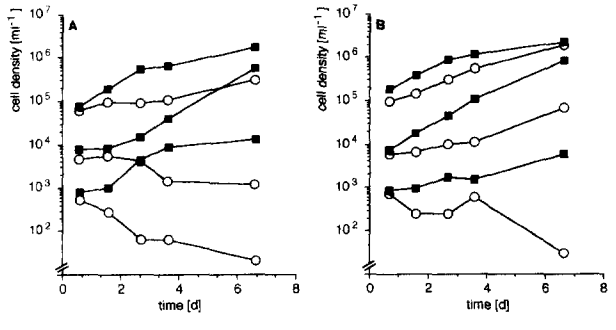

Fig. 2. Growth of serum-independent cell lines under DHFR-selective conditions. Comparison of the growth of SSF3 in T-flasks at passage 27 (A) and 82 (B) in medium containing glycine, hypoxanthine and thymidine (closed symbols) or *dhfr*-selective medium without these compounds (open symbols). Subcultures were initiated at three different cell densities.

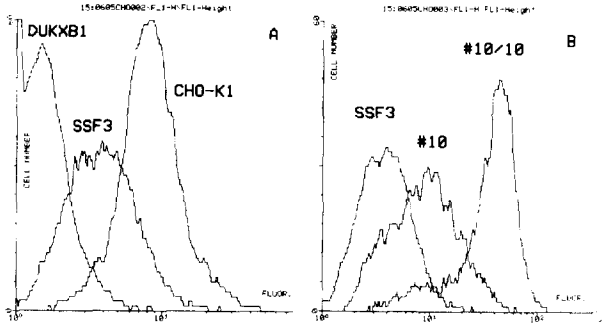

Fig. 3. FACS analysis of DHFR expression in transfected wild-type and serum-independent CHO cells. The intracellular DHFR was visualized by labeling the cells with FITC-methotrexate. Addition of an excess (1 μM) of non-fluorescent methotrexate suppressed fluorescence of SSF3 and CHO-K1 to the level of *dhfr*⁻ DXB11 cells (not shown), but had no effect on DXB11 fluorescence. (A–B) Comparison of different cell lines discussed in the text.

This suggests that a transfer of nutrients occurs from the *dhfr*-expressing cells to those lacking *dhfr*. The presence of DHFR enzyme activity in SSF cells could also be demonstrated by a spectrophotometric assay (Fig. 1) and could be inhibited completely with methotrexate. By this assay, later passages of SSF3 cells expressed about 10 times more DHFR enzyme activity than the *dhfr*⁻ CHO(dhfr⁻) parent cells and approximately 10 times less DHFR than wild-type CHO-K1 cells. By limited dilution cloning, sublines could been isolated of the SSF3 cell line with a very low percentage of *dhfr*⁺ cells. However, these lines always slowly reverted to a *dhfr*⁺ phenotype during prolonged culture under protein-free conditions. That growth under selection conditions was due to expression of a genuine DHFR enzyme could also be demonstrated by FACS analysis of cells treated with a fluorescent derivative of the DHFR inhibitor methotrexate [20]. SSF3 cells displayed a fluorescence intensity midway (on the logarithmic scale!) between that of standard CHO(dhfr⁻) cells and the wild-type parent cell line of CHO(dhfr⁻) cells, CHO-K1 (Fig. 3A).

3.5. *Adaptation of the dhfr selection procedure for SSF3-derived cell lines*

Because SSF3 cells express already some DHFR activity, culture conditions had to be found which would discriminate between untransfected cells with only this endogenous DHFR activity and *dhfr*-transfected cells with a higher activity. When hypoxanthine, thymidine and glycine was omitted from the medium, the bulk of untransfected SSF3 cells ceased to grow, but in mock transfection/selection experiments many apparent *dhfr*⁺ colonies were still obtained. When also 5 nM methotrexate was included, the *dhfr*⁺ colonies not containing a transfected *dhfr* gene were eliminated. The relationship between transfection efficiency and methotrexate concentration is shown in Fig. 4. At methotrexate concentrations higher than 5 nM, the selection behavior of the SSF3 cell line was not significantly different from that of the parental CHO(dhfr⁻) cell line, DXB11 (Fig. 4).

The approach described above was used to isolate clones transfected with pCGA72PCRK2, a plasmid combining a *dhfr* gene with a gene coding for the hybrid plasminogen activator K2tu-PA [11]. Only the transfection and the first 2 weeks of culture in Petri dishes were done in selective medium with 4% dialyzed FCS. Protein-free medium was used once the clones of transfected cells had been transferred into microwells (2 weeks after transfection). Essentially, all clones picked (90 out of 96) continued to grow when placed in protein-free medium. About 50% of these clones also expressed plasminogen activator.

3.6. *Amplification of dhfr-linked K2tu-PA gene in SSF3 cells*

Twelve K2tu-PA-producing clones, identified by ELISA [7] were subjected to selection in stepwise increasing concentrations of methotrexate. Of these, in at least six the production of the plasminogen activator was increased significantly after methotrexate selection. Measured by FACS analysis, the initial pCGA72aPCRK2 transformants expressed DHFR levels comparable to CHO-K1, which increased further after selection in 100 nM methotrexate (cell line 10/10, Fig. 3B). After selection using gradually methotrexate concentration, the expression of K2tu-PA increased in parallel to the increase of *dhfr* expression (Fig. 5).

3.7. *SSF3 cells produce single-chain recombinant plasminogen activator*

After activation in vitro with plasmin, no significant difference was seen in the specific enzymatic activity between purified SSF3-derived K2tu-PA and material derived from transfected standard CHO(dhfr⁻) cells [8–10]. However, 20–60% of the K2tu-PA from the conditioned medium of transfected CHO(dhfr⁻) cells (if cultured without addition of protease inhibitors) was found to be in the two-chain form and was already enzymatically active prior to deliberate activation with plasmin. In contrast, this was the case for less than 3% of K2tu-PA from

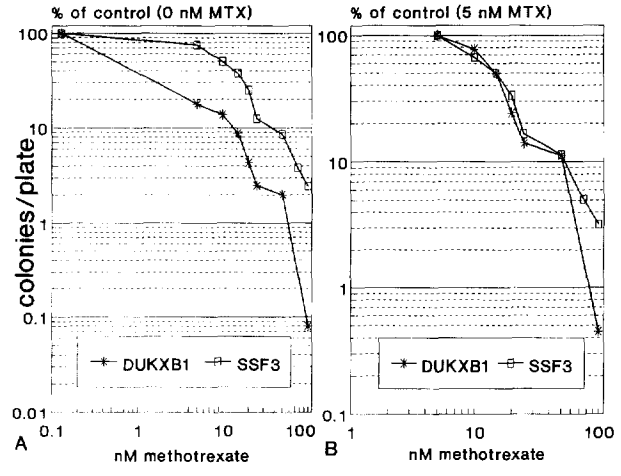

Fig. 4. Transfection efficiency and methotrexate resistance of SSF3 cells. Formation of *dhfr*⁺ colonies at different methotrexate concentrations after transfection of DXB11 and SSF3 cells with pCGA65e, a plasmid with dhfr in an SV40 'early' expression cassette [11]. The number of *dhfr*⁺ colonies obtained in selective medium without methotrexate (100% values) were 500 ± 20 and 800 ± 50 (n = 4) for DXB11 and SSF3, respectively. With SSF3, but not with DXB11 also *dhfr*⁺ colonies were obtained in a mock transfection experiment (600 ± 100 colonies, not subtracted from percentile values). Results are expressed (A) as a percentage of the number of colonies obtained in wselective medium without methotrexate or (B) expressed as percentage of the number of colonies obtained with 5 nM methotrexate. Note that above 5 nM there is effectively no contribution from the endogenous DHFR activity in the SSF3 cell line as the two curves practically coincide (panel B).

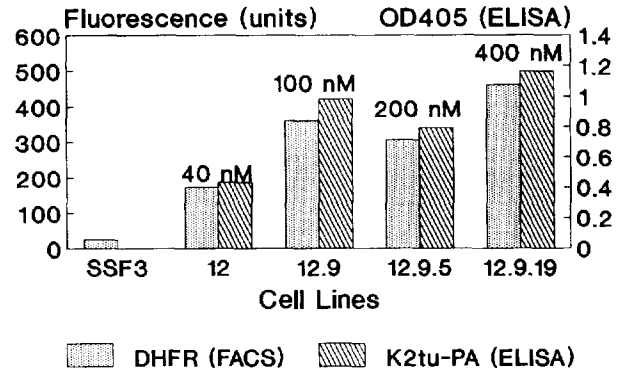

Fig. 5. Correlation between dhfr and K2tu-PA expression during amplification in serum-independent CHO cell lines. Comparison of expression of *dhfr* and K2tu-PA in SSF3 cells and four cell lines transfected with pCGA72aPCRK2 [11] all derived from clone 12, which were selected to grow in different concentrations of methotrexate (concentration indicated in nM above the bars).

transfected SSF3 cells, whereby 3% is the lower detection limit of the method used.

4. Discussion

4.1. Possible mechanisms of reversion of the dhfr⁻ genotype

In the creation of the CHO(dhfr⁻) cell line from CHO-K1 [16], both functional *dhfr* alleles were sequentially inactivated (Fig. 1). The first allele was inactivated creating the hemizygous line UKB25 from which in turn DXB11 was isolated [21]. As the results above show, for at least one of the genes inactivation is not irreversible. This is most likely the allele already inactivated in UKB25, because it was isolated after mutagenesis with ethyl methane sulfonate, a base substitution mutagen. The second allele was inactivated after gamma radiation, which is more likely to have induced an irreversible deletion. As DXB11 is the most commonly used CHO(dhfr⁻) mutant [1], it is important to be aware of the possibility of reactivation of the *dhfr* gene. DG-44, another *dhfr*⁻ CHO cell line, more stable but not as widely used, appears to have lost both DHFR alleles by deletions [22].

4.2. A connection between dhfr deficiency and serum requirement?

It is not entirely clear why, even when 10 mg/l of all four ribo-and deoxyribonucleosides are supplied, *dhfr*⁻ CHO cells should still revert to *dhfr*⁻ under protein-free conditions. Possibly, *dhfr*-deficient CHO cells require besides the nucleic acid precursors another intermediate metabolite which is supplied by the serum in standard media. This hypothesis is supported by observations made while testing batches of dialyzed serum used in standard *dhfr* selections. When the non-selective medium was supplemented with dialyzed serum, the proliferation rate of the CHO(dhfr⁻) cells was always slightly reduced compared with that observed with complete serum. In contrast, for the parental *dhfr*⁺ CHO-K1 or CHO(dhfr⁻) cells, which had been transfected with a DHFR gene, the growth-promoting activity of the dialyzed serum batches was equal and often better than with undialyzed serum. Possibly, the results obtained with the SSF cells are due to the absence of this/these metabolite(s) associated with the low-molecular weight serum fraction in the protein-free medium. One candidate for the unidentified substance would be *N*-formyl-methionine needed to initiate mitochondrial protein synthesis, which normally is synthesized via a DHFR-dependent pathway.

4.3. Prospects for animal cell culture in protein-free media

Elimination of serum as biological variable greatly facilitates development of more economic production processes. The absence of serum proteins eases purification of the recombinant proteins and avoids degradation of the recombinant product by serum proteases. The absence of two-chain K2tu-PA is a good example of the latter. Incidentally, these results probably exclude proteases released from the CHO cells themselves as the cause of formation two-chain plasminogen activator in CHO cell production systems. It was not necessary to use protease inhibitors in the medium or to include an additional purification step to eliminate the enzymatically active form. The highest levels of production of this and other recombinant proteins obtained with stably transfected SSF3-derived cell lines were, like observed with the parental CHO(dhfr⁻)-derived cells, around 20–50 μg per 10^6 cells day^{-1} or 2–5 10^{14} molecules cell^{-1} day^{-1} [23]. The availability of procedures to manipulate mammalian cells genetically in the absence of serum also opens the possibility of using these cells for other experiments than production of recombinant proteins, where serum-containing medium would interfere, for example in assays of signal transduction by hormones naturally present in serum.

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