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Transfection of DHFR$^-$ and DHFR$^+$ mammalian cells using methotrexate-resistant mutants of mouse dihydrofolate reductase

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Site-directed mutagenesis was used to generate mutants of mouse dihydrofolate reductase more resistant to methotrexate than the wild type enzyme. The mutant genes were used to transfect either DHFR$^-$ or DHFR$^+$ cell lines. These mutants, as well as the wild type gene, were able to confer methotrexate resistance to DHFR$^-$ CHO cells. The number of selected colonies decreased with increased concentrations of methotrexate. The number of colonies observed at 10 μM methotrexate is correlated with the K_i(MTX) of the enzyme: the higher the K_i, the higher the number of colonies for the corresponding mutant. In contrast, the transfections of DHFR$^+$ cells gave a few numbers of colonies not different for the wild type and the mutants.

Site-directed mutagenesis; Transfection; Mouse dihydrofolate reductase; DHFR$^-$ cells (CHO)

1. INTRODUCTION

Several dominant selective markers have been adapted for use in gene-transfer experiments [1]. Of particular interest for in vivo gene transfer is the gene coding for dihydrofolate reductase (DHFR; 5,6,7,8-tetrahydrofolate: NADP$^+$ oxidoreductase, EC 1.5.1.3). The folate analogue methotrexate (MTX) binds tightly to the catalytic site of the wild-type enzyme, resulting in a complete loss of DHFR activity at very low concentrations of MTX and thus, death of the cell. Obtention of a cDNA encoding an altered DHFR with an increased resistance to MTX might provide a dominant, metabolically selectable marker that could facilitate the introduction of foreign genes into a wide variety of mammalian cells. This marker has also an advantage in that it can be amplified [2], and amplification may be associated with increases in both copy number and expression of cotransferred genes [3].

Several mutant forms of DHFR have been identified in cultured cells that survive high doses of MTX [4–6]. We used site-directed mutagenesis to produce mutants of mouse DHFR more resistant to MTX than the wild-type enzyme [7]. We obtained a wide range of mutants with K_i's increased from 10^2 to 7.5×10^5-fold compared to that of the wild-type enzyme (0.004 nM). The two mutants exhibiting the highest K_i (MTX) and two other mutants obtained by in vivo selection in *B. subtilis* [8] were used to transfect mammalian cells with DHFR$^-$ or DHFR$^+$ phenotypes. A correlation could be established between the modified properties of the various mutants and their capacity to make the recombinant cells resistant to methotrexate.

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2. MATERIALS AND METHODS

Obtention of the mutants by site-directed mutagenesis, purification of the proteins and kinetic assays were performed as previously described [7].

2.1. *Transfection of eucaryotic cells*

DHFR$^-$ chinese hamster ovary (CHO) DXB11 [9], mouse L [10], and mouse teratocarcinoma PCC4 [11] cells were grown in Dulbecco's modified Eagle's medium supplemented with 8% fetal calf serum. Since they are DHFR$^-$ and Pro$^-$, for growing DXB11 cells the medium was supplemented with hypoxanthine, glycine, thymidine (HGT) and proline [9]. For transfection, 24 h after seeding 3×10^5 cells into 60 mm diameter tissue culture dishes, a calcium precipitate prepared with 5 μg of undigested plasmid DNA was added to the culture medium, and left 48 h [12]. The cells were then washed, dispersed, counted and plated in growth medium at 10^6 cells (for PCC4) or at 10^5 cells (for DXB11 and L) per 100 mm dish. After 24 h growth medium was replaced by selective medium, i.e. Dulbecco's medium lacking HGT for DXB11 cells in the absence or presence of methotrexate, or Dulbecco's medium for L and PCC4 cells supplemented with the desired concentration of methotrexate.

3. RESULTS AND DISCUSSION

3.1. *Inhibition of dihydrofolate reductase by methotrexate*

Table I lists the positions of the mutations obtained in mouse DHFR either by selection in bacteria in presence of methotrexate (Phe-31 -> Ser and Gln-35

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Table I

Kinetic parameters of dihydrofolate reduction

	K_i (MTX) (nM)	$K_m(H_2F)$ (μM)	V_{max} (min^{-1})
$DHFR^+$	0.004 ± 0.001	0.09 ± 0.02	360 ± 30
Phe→Ser 31	4.4 ± 1	4.5 ± 0.5	330 ± 30
Gln→Pro 35	175 ± 10	20 ± 2	n.d.[a]
Phe→Arg 31	190 ± 10	2.5 ± 0.5	60 ± 10
Leu→Arg 22	3000 ± 100	1 ± 0.2	6 ± 1

[a]Not determined

-> Pro) [8] or by site-directed mutagenesis (Phe-31 -> Arg and Leu-22 -> Arg) [7]. The inhibition constants (K_i) for methotrexate of the different enzymes as well as their kinetic parameters versus dihydrofolate, the substrate competing with methotrexate for the same binding site, are also listed in Table I. The K_m (H_2F) is increased for all mutants but to a lesser extent than the K_i for methotrexate. The V_{max} is decreased for Arg-31 and Arg-22 mutants which indicates a decreased efficiency of transformation of substrate to product. One constant which combines mutational effects on K_m's and K_i's is the I_{50}, the concentration of methotrexate which inhibits the reduction of dihydrofolate by 50%. Fig. 1 shows the inhibition curves which give the I_{50}, using crude extracts under defined experimental conditions (see legend). Under these conditions, the I_{50} is 3×10^{-9} M for the wild type enzyme and respectively 5×10^{-8} M, 4×10^{-7} M, 9×10^{-7} M and 3×10^{-5} M for Ser-31, Pro-35, Arg-31 and Arg-22 mutants. Arg-22 mutant exhibits the highest K_i and I_{50}. K_m for dihydrofolate of this mutant is not very much changed indicating an efficient binding but the V_{max} is dramatically decreased indicating a lowered activity.

3.2. *Construction of eucaryotic vectors*

The different DHFR cDNAs were cloned in the pSV2 eucaryotic expression vector as described in Fig. 2, to generate the respective pSV2-DHFR mutant plasmids as well as the pSV2-$DHFR^+$ used as a control. A unique Fnu4H1 site located 7 bases before the ATG of DHFR cDNA was used to join the coding sequence of DHFR to the early promoter of SV40. pSV2 vectors carrying various selective markers have been shown to be very efficient for transfection of numerous cell lines.

3.3. *Transfection of cells with the recombinant plasmids*

The kinetic parameters of the various mutants clearly indicated that they had presented different decreases for the binding of methotrexate (Fig. 1). It was interesting to test if the differences were correlated to the ability of the various mutants to confer a methotrexate resistance to eucaryotic cells. For that purpose, CHO $DHFR^-$ cells were transfected with either the wild-type or the mutant plasmid constructions and colonies were selected in the absence of HGT. Various concentrations of methotrexate were added to the selective medium at the initial time of the selection. Colonies were stained and counted two weeks after the selective medium had been applied and the results are shown in Table II. In the absence of methotrexate, the number of colonies is not identical from one plasmid to the other. This result can be attributed to the thermal stability of the mutant proteins. We have previously demonstrated [7] that the

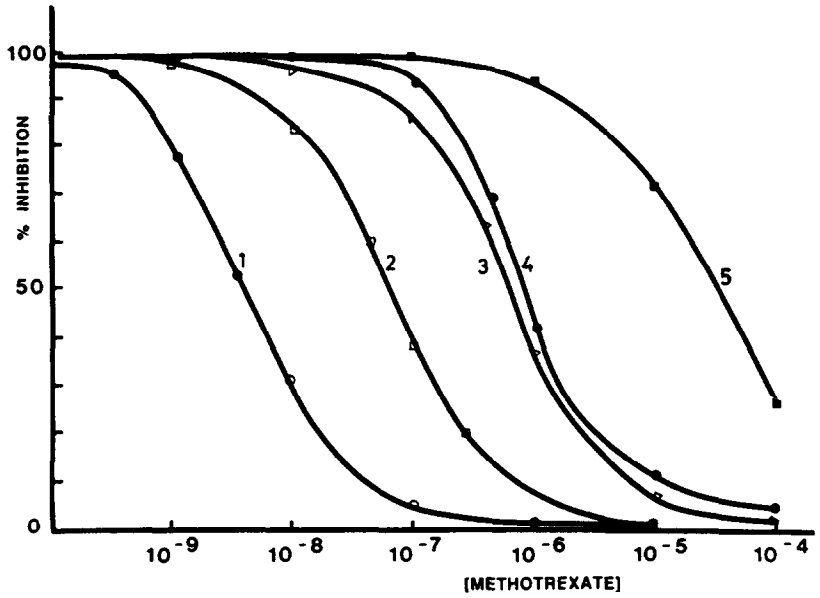

Fig. 1. Measurement of I_{50} on crude extracts. DHFR was assayed spectrophotometrically as follows: 100 μM NADPH, 100 mM potassium phosphate, pH 7.9, 100 mM potassium chloride, in a total volume of 1 ml. Incubation time in presence of methotrexate was 10 min at 25°C and the reaction was initiated by addition of 50 μM H_2F. Percent inhibition was calculated from an assay without methotrexate. 1, Wild-type; 2, Ser-31; 3, Pro-35; 4, Arg-31; 5, Arg-22.

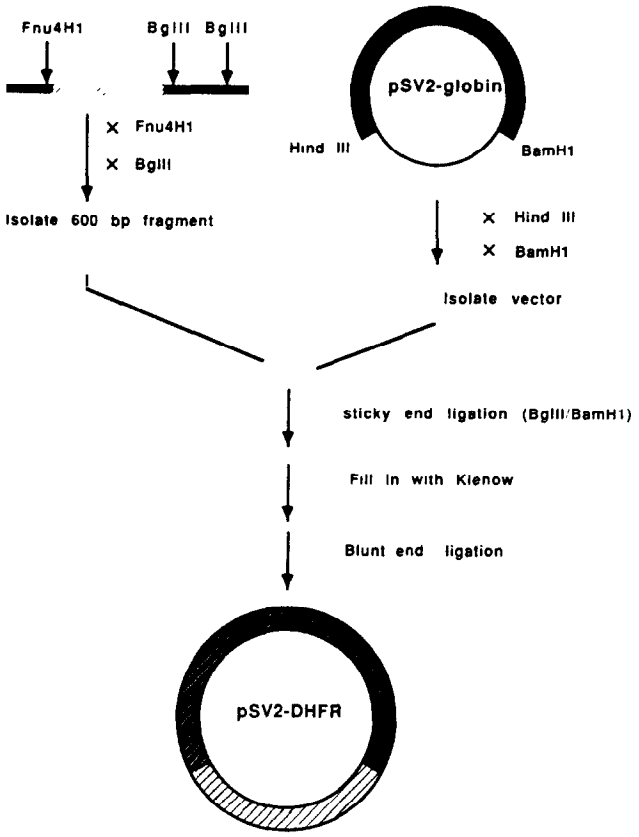

Fig. 2.Construction of DHFR eucaryotic expression plasmids. Dark dahsed lines, pSV2 vector; dark lines, pUC8 sequences; dashed lines, DHFR sequences.

Pro-35 protein was less stable while Arg-31 and Arg-22 were more stable than the wild type enzyme at 37°C. Ser-31 has the same stability as the wild type enzyme. The low efficiency of Pro-35 can be due to a lower amount of protein present in the cell after the transfection and vice-versa for Arg-31 and Arg-22.

In the presence of methotrexate, colonies obtained after transformation by pSV2-DHFR mutants were able to propagate in much higher levels of methotrexate than colonies derived from transfections with pSV2-DHFR$^+$, with the exception of Pro-35 for the reasons mentioned above. Moreover, the number of colonies obtained increased according to the K_i (MTX) for each mutant: the higher the K_i, the higher the number of colonies.

1 μM methotrexate is largely above the concentration which is toxic for wild-type mammalian cells. At that methotrexate concentration, the pSV2-DHFR$^+$ plasmid still gave rise to a large number of colonies. This apparent discrepancy might be explained by the presence of a larger amount of DHFR in the transfected cells than in a wild-type DHFR$^+$ cell. Such a hypothesis is difficult to check by direct assay of DHFR activity in cellular extracts since the activity is almost undetectable by spectrophotometric assay in wild type cells: 1.25 ± 0.25 μmol THF/min per mg (units) compared to the background: 1 ± 0.2 units. However, the activity of transfected cells is slightly above the background in (2.7 ± 0.3 units), possibly because the number of integrated copies is high in transfected cells (data not shown).

Table II

Transfection efficiencies of the different pSV2-DHFR plasmids in DHFR$^-$ CHO cells

[MTX]	DHFR$^+$	Ser-31	Pro-35	Arg-31	Arg-22
0	370 ± 30	290 ± 30	100 ± 20	>1000	>1000
100 nm	165 ± 20	142 ± 20	50 ± 10	>1000	>1000
1 μM	26 ± 10	107 ± 20	20 ± 15	250 ± 30	350 ± 30
10 μM	0	10 ± 5	0	60 ± 10	280 ± 30

Since the mutant plasmids were able to convert DHFR$^-$ to DHFR$^+$ cells capable of growing at methotrexate concentrations sufficient to suppress the endogeneous enzyme activity of wild-type cells, we explored the use of the mutant cDNAs as dominant selective markers in cells producing normal levels of unaltered DHFR. The concentration of methotrexate necessary to kill non transfected cells is 200 nM for L cells and 50 nM for PCC4. Transfection experiments were performed under the same conditions as for DHFR$^-$ CHO cells but using 300 nM MTX for L cells and 80 nM for PCC4 cells. In 10 different experiments, the frequency of colonies obtained with mutant plasmids was very low compared to the frequency obtained with DHFR$^-$ CHO as well as to the frequency obtained in control experiments with another selective marker such as pSV2-gpt. Moreover, most of the colonies did not survive after transfer. These results were different from those obtained by Simonsen and Levinson [13]. We have tried to reproduce exactly their experiments, i.e. applying the selective medium after 48 h (instead of 24 h), staining the colonies after 10 days (instead of two weeks), and using the pFR400 plasmid (containing the Arg-22 mutant cDNA as in our case but with a different 3′ end regulatory region). These modifications did not improve the results. A higher number of colonies was detected by staining at day 10 but they were not viable after transfer.

We can interpret the different results obtained with DHFR$^-$ CHO and DHFR$^+$ cell lines as follows: in case of DHFR$^+$ cells, if the catalytic activity or the K_m for dihydrofolate is too much modified, as it was the case for all the mutants examined, there is no selective advantage given by the transfected cDNA versus the wild-type enzyme already present in the cell. If the actrivity of the endogenous enzyme is limited by the availability of the substrate concentration, a variant enzyme capable of binding substrate but with a very low efficiency of conversion to product, could be considered as an inhibitor of the endogenous DHFR enzyme. In that case, even if the affinity of the mutant for MTX is greatly decreased, this is not a selective advantage. This conclusion was also reached by Gordon [14] who con-

structed transgenic mice with the Arg-22 mutant. The mice exhibited developmental anomalies identical to those observed upon administration of thymidylate synthetase inhibitors to fetal rats. This enzyme produces dihydrofolate and thus, its inhibition leads to a decrease of intracellular pool of this compound [15].

4. CONCLUSION

Wild-type mouse dihydrofolate reductase cDNA has been proven to be very efficient to transfect DHFR$^-$ cells and to co-amplify an associated gene [3]. Mutants of mouse DHFR can also transfect these cells, sometimes with a higher efficiency than the wild type DHFR (Table II). However, the selective advantage of an increased resistance to MTX is not real when wild type cells are used in which they rather behave as simple competitive inhibitors of the endogeneous enzyme.

REFERENCES

[1] Kucherlapati, R.R. and Skoultchi, A.I. (1986) CRC Critical Review in Biochemistry 16, 349–379.
[2] Alt, F.W., Rodney, E.K., Bertino, J.R. and Schimke, R.T. (1978) J. Biol. Chem. 253, 1357–1370.
[3] Lau, Y.F., Lin, C.C. and Kan, Y.W. (1984) Mol. Cell Biology 4, 1469–1475.
[4] Melera, P.W., Davide, J.P., Hession, C.A. and Scotto, K.W. (1984) Mol. Cell Biology 4, 38–48.
[5] Melera, P.W., Davide, J.P. and Oen, H. (1988) J. Biol. Chem. 263, 1978–1990.
[6] Haber, D.A., Beverley, S.M., Kiely, M.L. and Schimke, R.T. (1981) J. Biol. Chem. 256, 9501–9510.
[7] Thillet, J., Absil, J., Stone, S.R. and Pictet, R. (1988) J. Biol. Chem. 263, 12500–12508.
[8] Grange, T., Kunst, F., Thillet, J., Ribadeau-Dumas, B., Mousseron, S., Hung, A., Jami, J. and Pictet, R. (1984) Nucleic Acids Res. 12, 3585–3601.
[9] Urlaub, G., Kas, E., Carothers, A.M. and Chasin, L.A. (1983) Cell 33, 405–412.
[10] Sanford, K.K., Early, W.R. and Likely, G.D. (1948) J. Natl. Cancer Inst. 9, 229–246.
[11] Jacob, H., Boon, T., Gaillard, J., Nicolas, J.F. and Jacob, F. (1973) Ann. Microbiol. 124B, 269.
[12] Thillet, J., Kunst, F., Lasserre, C., Bucchini, D., Pictet, R. and Jami, J. (1984) Exp. Cell Res. 151, 494–501.
[13] Simonsen, C.C. and Levinson, A.D. (1983) Proc. Natl. Acad. Sci. 80, 2495–2499.
[14] Gordon, J.W. (1986) Mol. Cell Biol. 6, 2158–2167.
[15] Danenberg, P.V. (1977) Biochim. Biophys. Acta 473, 73–92.