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# Modification of glucose and glutamine metabolism in hybridoma cells through metabolic engineering

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**Abstract**

The present work describes the genetic modification of a hybridoma cell line with the aim to change its metabolic behaviour, particularly reducing the amounts of ammonia and lactate produced by the cells. The cellular excretion of ammonia was eliminated by transfection of a cloned glutamine synthetase gene. The metabolic characterisation of the transformed cell line includes the analysis of the changes introduced in its intracellular metabolic fluxes by means of a stoichiometric model. Furthermore, the reduction of lactate accumulation was attempted through an antisense mRNA approach, aiming to generate a rate limiting step in the glycolytic pathway, thus lowering the glucose consumption rate. The physiological results obtained with the transformed cells are discussed. A maximum reduction of about 47% in the glucose consumption rate was obtained for one of the transformations. However a main drawback was the lack of stability of the transformed cells

*Abbreviations:* gpt – xanthine-guanine phosphoribosyltransferase; GS – glutamine synthetase; TCA cycle – tricarboxylic acid cycle

## Introduction

Hybridoma cells, like all tumour cells, can be considered energetically efficient systems, since they are able to gain energy from different sources such as glucose and glutamine. This efficiency can be reached when cells are exposed to an extremely controlled environment such as a body tissue. The efficiency is lowered when glucose and glutamine concentrations are much higher than the physiological levels, as usually occurs when cells are cultured *in vitro*. In these conditions cells consume both substrates at high rates leading to the accumulation of lactic acid and ammonium up to inhibitory levels for cell growth (Ozturk et al., 1992).

The minimisation of the accumulation of lactic acid and ammonia is essential to obtain high cell densities in batch and fed-batch cultures of mammalian cells. This minimisation can be achieved following several strategies such as substrate substitution, optimisation of culture conditions or metabolic pathway engineering. Substitution of glucose by other carbon sources such as galactose (Glacken et al., 1989), fructose (Duval et al., 1992) or mannose (Jayme, 1991) can be used to reduce the rate of lactate production. However, it has been reported (Moellering et al., 1990) that complete glucose substitution by other hexoses can alter product glycosylation. The elimination of glutamine has also been assayed using glutamine dipeptide derivatives (Christie and Butler, 1994; Holmlund et al., 1992; Minamoto et al., 1991; Roth et al., 1988; Butler and Christie, 1994) or glutamine independent clones (Birch et al., 1994). The optimisation of culture conditions can be achieved modifying the temperature (Sureshkumar and Mutharasan, 1991; Reuveny et al., 1986; Chuppa et al., 1997) or using fedbatch techniques (Ljunggren and Haggstrom,

1990; Glacken et al., 1986). Both methods reduce lactate and/or ammonia production, improving culture performance. Transfection of the glutamine synthetase (GS) gene (Cockett et al., 1990; Bell et al., 1995; Birch et al., 1994; Bebbington et al., 1990) is up to now the most widely used strategy to alter metabolic pathways. Cells transformed with the GS gene can grow in media supplemented with glutamate instead of glutamine, with a direct elimination/reduction of ammonia generation, either by glutamine decomposition or metabolism.

The observation that the complementary interaction of nucleotide sequences occurs in nature for the regulation of cellular functions, led to the notion of 'antisense' as a biological entity (Kruger et al., 1982). The term antisense defines any DNA or RNA that inhibits gene function by mechanisms that require complementary base-pairing to the genetic target. In the last 15 yr, it has been shown that the expression of antisense RNA as well as exogenous introduction of antisense DNA can affect a variety of cell functions (Scanlon et al., 1991; Walder and Walder, 1988). It is generally assumed that the mechanism of inhibition by nuclear-derived antisense RNA entails sequence-specific hybridisation of antisense RNA transcripts to the target mRNA in the nucleus, thus blocking protein synthesis (Scherczinger et al., 1992). Under optimal conditions an antisense RNA approach could completely inhibit protein synthesis. Therefore, the reduction of the flux of a given metabolic reaction can be induced by the introduction of an antisense RNA that decreases the amount of a given enzyme catalysing such reaction.

The present work is based on the fact that hybridoma cells consume glucose and glutamine much faster than required to maintain cell growth. To modify this trend, metabolic engineering strategies have been applied with the objective to reduce the rates of glutaminolysis and glycolysis and verify the feasibility of this approach. Modification of glutaminolysis has been achieved following transfection and expression of a cloned glutamine synthetase gene. In addition, modification of glycolysis has been studied by means of antisense RNA techniques directed to lower the glycolytic rate in two different stages: transport of glucose into the cell and the metabolic step catalysed by the enzyme enolase, responsible for the conversion of 2-phosphoglyceric acid to phosphoenolpyruvic acid.

## Materials and methods

*Cell line:* The KB-26.5 murine hybridoma was used throughout the work. This cell line produces an $IgG_3$ monoclonal antibody directed against the antigen $A_1$ of red cells. This cell line was kindly provided by Laboratorios Knickerboker SAE.

*Media:* The basal medium used was DMEM (Life Technologies) supplemented with sodium bicarbonate (3.7 g $l^{-1}$), phenol red (15 mg $l^{-1}$), $\beta$-mercaptoethanol (0.391 mg $l^{-1}$), insulin (0.836 mg $l^{-1}$) and 1% foetal calf serum (Biological Industries), and also with 25 mM glucose and 6 mM glutamine.

*Culture systems:* Cultures were performed in spinner flasks of 250 ml capacity, swirling at 40 rpm in a humidified atmosphere containing 5% $CO_2$ at 37 °C. Antisense experiments were carried out in 25 $cm^2$ T-flasks holding 10 ml of working volume and using inoculums of $1.0 \times 10^5$ cells/ml.

*Analytical methods:* Total and viable cell concentrations were determined by the trypan blue exclusion method using a Neubauer improved haemocytometer. Glucose and lactate concentrations were mesured with a YSI 2700 automated glucose and L-lactate analyser. Ammonium concentration was determined by a flow injection analysis system as described previously (Campmajó et al., 1994). Glutamine and other amino acid concentrations were measured by HPLC (Hewlett Packard 1090) using a reverse phase column (Aminoquant $200 \times 2.1$ mm) after derivatisation of the samples with OPA and FMOC for the analysis of both primary and secondary amino acids (Sanfeliu et al., 1996).

### *Specific consumption/production rates of extracellular metabolites*

The values of specific growth rate, and specific consumption/production rates for all the extracellular metabolites in the exponential growth phase were determined by a least squares fitting of the following expressions (Glacken et al., 1988):

$$X = X_0 \exp(\mu t)$$

$$C = C_0 + \frac{q_c 10^{-3} X_0}{\mu} \left[\exp(\mu t) - 1\right]$$

where $X$ is the cellular concentration in $10^6$ cells/ml, $\mu$ is the specific growth rate in $h^{-1}$, $C$ is the concentration of the compound studied in mM, and $q_c$ is its specific uptake/production rate in nmol/(h $10^6$ cell). The subscript 0 means the value at the start of the exponential growth.

*Plasmids construction*

*Glutamine synthetase:* The recombinant plasmid pCMGS.gpt (Bebbington et al., 1990) containing the GS cDNA under the control of the strong hCMV-MIE promoter was used. This plasmid contains a xanthine-guanine phosphoribosyltransferase (gpt) gene as the selectable marker and was kindly provided by Lonza Biologics Ltd.

*Antisense constructions:* The antisense and sense fragments for the rat brain glucose transporter GLUT1 were released from the recombinant plasmid prGT3 (Birnbaum et al., 1986) after restriction with *Bam*HI and purification of the resulting 436 bp fragment proximal to the ATG codon.

The antisense and sense fragments for the $\alpha$-enolase were obtained by PCR amplification of a cDNA library from adult mouse testis. Specific primers (20-mers) were designed to amplify a 408 bp fragment encompassing positions 104 to 511 of the reported mRNA (Kaghad et al., 1990)

Purified GLUT1 and enolase fragments were blunt-ended and cloned into the *Eco*RV site of pcDNA3 (Invitrogen) containing the Neomycin resistance gene, and their orientations identified by restriction analysis and agarose gel electrophoresis.

All recombinant DNA manipulations were carried out by standard procedures (Ausubel et al., 1997) and conducted in accordance with established guidelines for recombinant DNA research.

*Transfection and selection methods:* The liposome-mediated method (DOTAP, Boehringer Mannheim) was used to transfer the recombinant plasmids into KB-26.5 cells.

GS clones were selected by serial dilution using glutamine-free DMEM medium during 2–3 weeks.

GLUT1 and $\alpha$-enolase antisense and sense clones were selected in DMEM medium containing 500 $\mu$g/ml of Neomycin. This level was previously established as lethal for untransfected cells.

*RNA isolation and detection:* Total RNA was purified from 10 ml cultures containing $10^7$ cells, following the procedure described by Chirgwin et al. (1979). Polyadenilated RNA was fractionated with oligo(dT)cellulose (mRNA separator Kit, Boehringer Mannheim) and spotted onto Nylon Hybond-$N^+$ (Amersham). RNA dot blot analyses were performed using fluorescein-labelled partial cDNA probes for glucose transporters GLUT1 to GLUT5.

## Results and discussion

Continuous culture experiments performed previously to this work (Sanfeliu et al., 1997) have shown that the original KB-26.5 cell line consumes more glucose and glutamine than necessary to maintain cell growth. In continuous cultures performed at D = 0.0256 $h^{-1}$, a constant level of cells of $0.5 \times 10^6$ cells/ml could be maintaned over a wide range of glutamine consumption rates ranging from 102.4 up to 30.7 nmols/(h $10^6$ cells). The same trend could be observed for glucose consumption that ranged from 255.5 up to 76.8 nmols/(h $10^6$ cells). Since the cell concentration remained constant in all these conditions, it was concluded that there is no a real requirement to consume such large amounts of glucose and glutamine in order to maintain cell growth.

In an attempt to explain why the excess of glucose and glutamine consumed is excreted as lactate and ammonium ions a stoichiometric model was developed (Paredes et al., 1998). In brief the model consists in the derivation of a mass balance for each considered metabolite including the transport rates across membranes and their production or consumption rates in the intracellular reactions considered. All these data in conjunction with the measured specific uptake/production rates for all the extracellular metabolites, makes feasible to estimate the intracellular fluxes using a least squares procedure.

The data obtained with the metabolic flux modelization enabled to establish a hypothesis to explain the observed behaviour of these cells assuming a low efficiency of the malate-aspartate shuttle in KB-26.5 cells (Sanfeliu et al., 1997). This shuttle is the transport system that enables mitochondrial reoxidation of the NADH excess produced in the glycolytic pathway. If the electron-transport chain is inefficient the only way to regenerate cytoplasmic NADH is resorting to the lactate dehydrogenase pathway that uses pyruvate as a substrate. Thus, the amount of pyruvate that can be incorporated into the tricarboxylic acid cycle (TCA

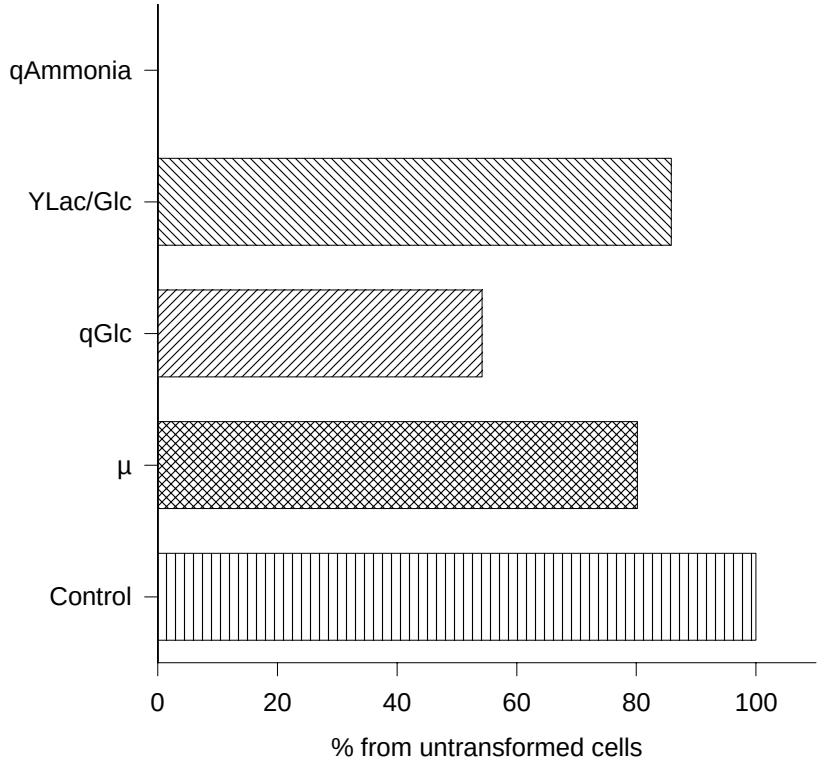


*Figure 1.* Physiological effects of GS transfection in KB-26.5 cells. Untransfected KB-26.5 cells were used as control. $\mu$ is the specific growth rate, qGlc indicates the specific glucose uptake rate, YLac/Glc is the molar yield of lactate-glucose and qAmmonia is the specific ammonia production rate.

cycle) is very small and the rapid glutaminolysis rate collapses and unbalances the cycle. Simultaneously, the large amount of ammonia generated from glutamine and other amino acid deaminations overflows cell capacity and is excreted as free ammonia, alanine and proline. Moreover since alanine, which is formed from pyruvate and glutamate, is produced in large amounts, the availability of pyruvate for the cell metabolism decreases.

This analysis of the hybridoma metabolism suggests that important re-distribution of cellular metabolism could be expected by modulating (i.e. decreasing) the rate of the two main metabolic pathways: glycolysis and glutaminolysis.

*Modification of glutamine metabolism*

As mentioned in the introductory section, the first genetic modification of the cell line KB-26.5 was the transfection with the glutamine synthetase gene. For optimal growth of the transfected cells the addition of alanine to 2.5 mM in the culture medium was required. The physiological consequences of the GS expression when cultured in batch mode are summarised in Figure 1. The transfected cells showed a lower growth rate (82%) than that of the untreated cells. This result can be due to the genetic modification, although it may reflect the specific growth rate of the clone transfected. This point still remains an open question. The most notable physiological consequence was the complete elimination of ammonia production. Similar effects have also been reported in the literature (DiStefano et al., 1996; Robinson, Chan et al., 1994; Bell et al., 1995). Another interesting point was a 50% reduction in the specific glucose uptake rate, although the lactate/glucose molar yield decreased only slightly. These results corroborate the interaction between the two main pathways in the hybridoma metabolism.

The data obtained in this physiological study were analysed by means of the stoichiometric model previously described (Paredes et al., 1998), conveniently modified to account for the GS reaction. The results obtained in the estimation of the corresponding intracellular fluxes are presented in Figure 2, both for the parental and GS transfected cells.

The analysis of the results from the GS trans-

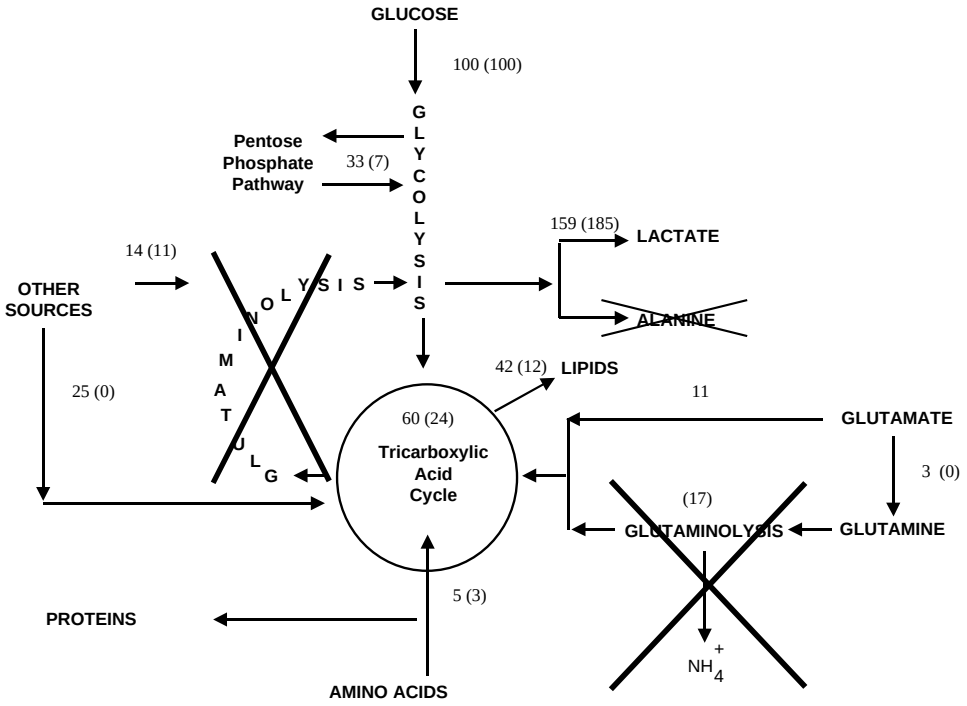


*Figure 2.* Schematic metabolic fluxes for the parental and GS transfected KB-26.5 cells. Values inside parentheses correspond to untransformed cells. Value shown are in relative units as a percentage of the specific uptake rate.

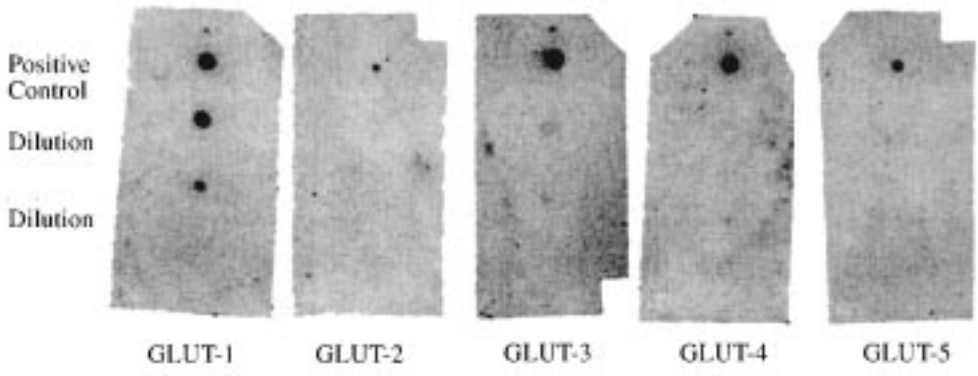


*Figure 3.* Identification of the glucose transporter for the KB-26.5 cell line.

formed cells show important metabolic changes with respect to non-transformed cells. Indeed, ammonia excretion becomes completely prevented. Likewise, the excretion of other nitrogenated compounds, such as alanine, does not occur in transformed cells. This event can be related to the second main metabolic change observed in the cells, that is, the significant reduction (almost 50%) of the glucose uptake rate. These were experimental evidences, obtained from the different analysis, and were also well incorporated into the model. These results evidence the interaction among the different pathways in the cell metabolism. A possible explanation for these results is that under culture conditions generating ammonia (such as non-transformed cells growing on glutamine), different glycolytic intermediates are consumed by the cell to produce nitrogenated end metabolites, such as alanine or proline, to reduce the internal ammonium concentration. When cells are cultured under conditions that generate low ammonium levels (such as GS transformed cells growing on glutamate), the requirement for glycolytic products becomes lower,

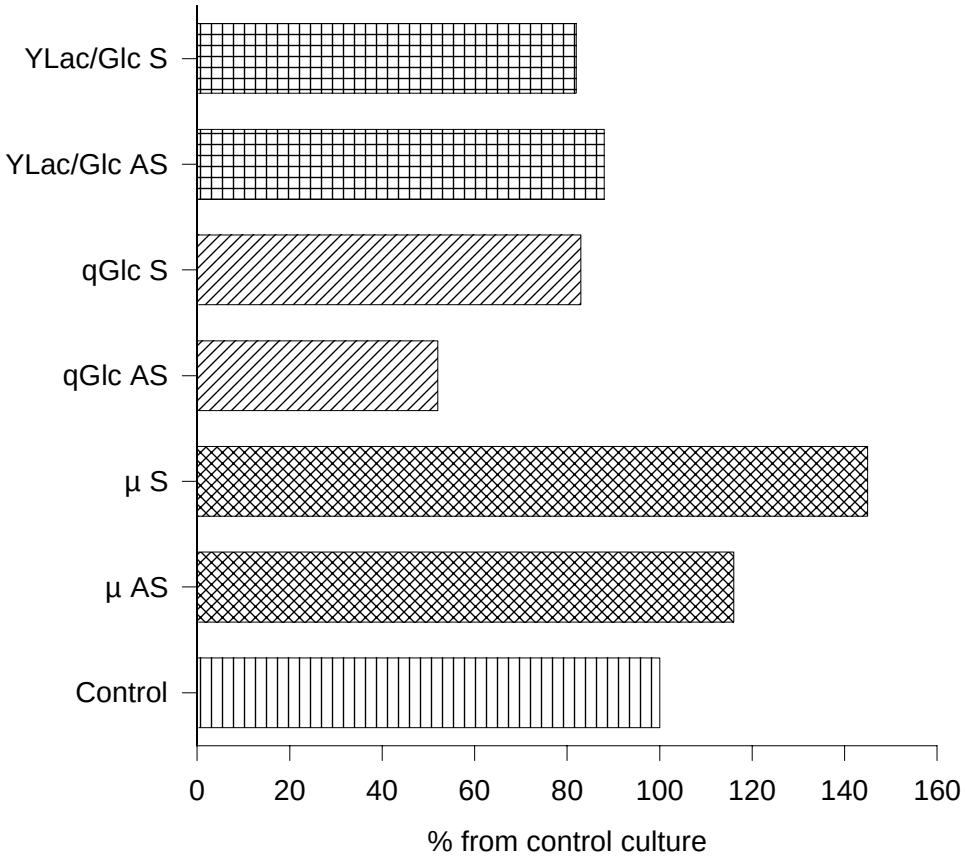


*Figure 4.* Physiological effects of sense and antisense constructions of GLUTI. Cells transformed with non-recombinant pcDNA3 plasmid were used as control. AS indicates antisense orientation of the cloned GLUT1 fragment and S the direct orientation. $\mu$: values corresponding to the specific growth rate. qGlc: values for the specific glucose uptake rate. YLac/Glc: molar lactate/glucose yield.

and the glucose uptake rate does too. On the other hand, the yield of lactate production with respect to glucose consumption remains almost unchanged in both types of cells. This indicates that the alternative mechanism of NADH regeneration through lactate formation remains essentially unchanged after cellular transformation. As regards to the TCA cycle, it can be suggested that the reduction in the glutaminolysis rate in the transformed cells prevents its collapse.

Modification of the glycolytic pathway As mentioned in the introduction, the direct modification of the glycolytic pathway was attempted using an antisense RNA approach directed to two different targets: the glucose transporter and the enzyme $\alpha$-enolase.

Several isoforms of the glucose transporter involved in the facilitated co-transport mechanism have been characterised in different animal cells (Gould et al., 1991; Birnbaum et al., 1986; Birnbaum, 1989; Carruthers, 1990; Gould and Bell, 1990).

To identify the specific glucose transporter expressed in the KB-26.5 cells, a series of RNA dot-blots were treated with chemiluminescent partial cDNAs of the 5 most common glucose transporters. The analysis revealed that GLUT1 is the transporter for glucose in KB-26.5 cells as evidenced in the dot-blots presented in Figure 3.

The physiological effects of the transformation of the hybridoma cells with these constructions are presented in Figure 4 for GLUT1 and in Figure 5 for $\alpha$-enolase. All transfected cells show higher growth rates than the control culture. This can be explained assuming that integration in the genome occurs randomly promoting a random effect. As a consequence, cells carrying either antisense or sense constructions might have some advantage over untransformed cells.

In the above mentioned conditions the specific glucose uptake rate decreases about 22% for the antisense enolase construction, 48% for the antisense GLUT1, 17% for the sense GLUT1 whereas no significant effect for the enolase sense construction was observed.

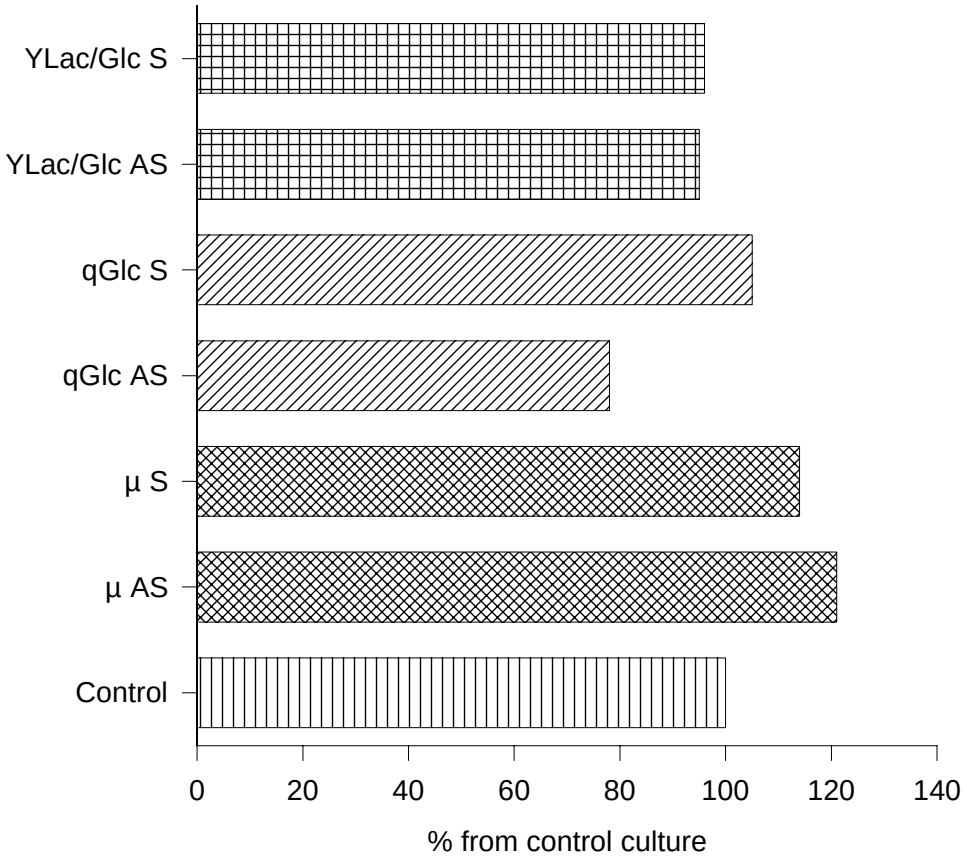


*Figure 5.* Physiological effects of sense and antisense constructions against $\alpha$-enolase. Cells transformed with non-recombinant pcDNA3 plasmid were used as control. AS indicates antisense orientation of the cloned $\alpha$-enolase fragment and S the direct orientation. $\mu$: values corresponding to the specific growth rate. qGlc: values for the specific glucose uptake rate. YLac/Glc: molar lactate/glucose yield.

From these values one can conclude that it is likely that both antisense constructions are able to generate a rate-limiting step in the glycolytic pathway. However, the effect of GLUT1 sense construction underlines the fact that the mechanism of antisense inhibition is poorly understood. This effect is documented in the literature (Cameron and Jennings, 1991; Van der Krol et al., 1990; Napoli et al., 1990; Smith et al., 1990) and known as 'sense co-suppression' (Scherczinger et al., 1992). Although the reasons for this effect are unclear, several explanations have been proposed and reviewed in Scherczinger et al. (1992). It seems possible that the presence of extra gene copies in the nucleus may be sufficient to promote co-suppression through some interaction of the genes or titration of nuclear factors. The molar yield for lactate/glucose remains almost unchanged for the enolase constructions. However, the GLUT1 constructions show 12% decrease for antisense construction and 18% for the sense construction.

An important drawback is that the constructions described here are unstable for the culture conditions used and it was impossible to isolate a clonal population. Although the selective pressure was maintained, the specific growth rate, the specific glucose uptake rate and the lactate/glucose yield after a certain number of passages of the cells in the culture medium went back to the level of the untransfected cells. This is exemplified by the evolution of the specific glucose uptake rate shown in Table 1. The values presented in Figures 4 and 5 were obtained after 28 days of transfection. At this time a 96 h. long T-flask experiment was carried out and the specific rates for growth rate, glucose uptake and lactate production were determined as decribed in the materials and methods section.

To confirm the results discussed above, a second transformation with the same set of plasmids was carried out. Although numerically different, the results showed identical trends and similar instability. In this

*Table 1.* Specific glucose uptake rate evolution. Values are shown as% of intact pcDNA3. The first column shows times after transfection. Each value is the average from four consecutive glucose measurement on a daily basis. GLUT1 S refers to the cells transfected with the sense construction for GLUT1 glucose transporter, whereas GLUT1 AS is the antisense construction. Enolase S refers to the cells transfected with the sense construction for $\alpha$-enolase, whereas Enolase AS is the antisense construction. pcDNA3 refers to the cells transfected with non-recombinant pcDNA3.

| time (d) | GLUT1 S | GLUT1 AS | Enolase S | Enolase AS | pcDNA3 |
|---|---|---|---|---|---|
| 28 | 83 | 52 | 105 | 78 | 100 |
| 36 | 93 | 119 | 96 | 91 | 100 |
| 47 | 93 | 92 | 98 | 99 | 100 |

regard it may be concluded that although the use of antisense constructions for GLUT1 and enolase to modify the glycolysis rate is feasible, further work must be carried out to stabilize the antisense effect and to study the sense co-suppression.

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