THE ASSOCIATED REACTIVATION OF TWO X-LINKED GENES

THE SPONTANEOUS AND AZACYTIDINE-INDUCED RE-EXPRESSION OF ORNITHINE TRANSCARBAMOYLASE AND GLUCOSE-6-PHOSPHATE DEHYDROGENASE IN A RAT HEPATOMA

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SUMMARY

Two X-linked genes, specifying ornithine transcarbamoylase (OTC) and glucose-6-phosphate dehydrogenase (Glc-6-PD), are reversibly suppressed in certain derivatives of the rat H4-II-E hepatoma. Either gene can become reactivated spontaneously, and it is shown that both can be reactivated by azacytidine treatment. This gene reactivation has been investigated by enzyme assay and by the use of selective growth media ('ornithine-medium' to select for OTC, and medium containing diamide to select for Glc-6-PD). There is a clear tendency for both genes to be reactivated together, though either can become active alone. Since OTC is an enzyme of the urea-cycle, and Glc-6-PD is an enzyme of the hexose monophosphate shunt, and since these two pathways are normally under quite separate control, it would seem that the coupled regulation of the two genes in these hepatomas is abnormal. It is suggested that the suppression of the two genes resembles X-inactivation: in both cases, azacytidine treatment induces gene reactivation with a high frequency and results in different clones of cells expressing widely varying amounts of enzyme activity. The association between the re-expression of OTC and Glc-6-PD might indicate that some phenomenon like the position-effect is occurring.

INTRODUCTION

Ornithine transcarbamoylase (OTC) is one of the urea-cycle enzymes required for the conversion of ornithine to arginine. The presence of these enzymes in hepatocytes and some hepatomas enables such cells to grow in medium containing ornithine in place of arginine, i.e. 'ornithine medium' (Leffert & Paul, 1972; Niwa, Yamamoto & Yasumura, 1979; Goss, 1984a). Certain derivatives of the rat hepatoma H4-II-E, for example Fu5 and Fu5-TG1, cannot grow in ornithine-medium because they have only trace amounts of OTC (Widman, Golden & Chasin, 1979; Niwa et al. 1979; Goss, 1984a). However, if these hepatomas are plated in ornithine-medium, they spontaneously give rise to progressively growing clones with a frequency of the order of $10^{-3}$ to $10^{-4}$. These clones are found to express high levels of OTC (Goss, 1984a). The relative ease with which these hepatomas regain their OTC suggests that their initial deficiency is likely to be the result of some regulatory phenomenon rather than a mutation. The same hepatomas also lack another enzyme, glucose-6-phosphate dehydrogenase.

Key words: hepatoma, urea-cycle, ornithine utilization, diamide, azacytidine, ornithine transcarbamoylase, glucose-6-phosphate dehydrogenase.
dehydrogenase (Glc-6-PD), which, like OTC, can sometimes be spontaneously regained (Pitot, Peraino, Morse & Potter, 1964; Deschatrette & Weiss, 1974; Croce, Litwack & Koprowski, 1974; De Luca & Matheisz, 1976). The mechanisms and the significance of these two superficially similar phenomena remain to be elucidated.

This paper reports a study of the re-expression of OTC and Glc-6-PD in the H4 derivatives Fu5 and Fu5-TG1. Extensive use has been made of selective systems for both enzymes: selection for OTC is described above, selection for Glc-6-PD can be achieved by exposing the cells to the glutathione-oxidizing agent ‘diamide’ (i.e. 1,1’-azobis(N,N-dimethylformamide) (Kosower & Kosower, 1969). Cells can withstand diamide only if they have an active hexose monophosphate pathway capable of producing NADPH (reduced nicotinamide adenine dinucleotide phosphate), which in turn maintains cellular glutathione in a reduced state (Rosenstraus & Chasin, 1977).

It was suggested that it might be interesting to make a simultaneous investigation of OTC and Glc-6-PD, by a chance observation made during the course of experiments on another H4 derivative, Ø1. This cell line had at some stage regained OTC expression (Goss, 1984a), and it was further noted that it has also regained Glc-6-PD (S. J. Goss, unpublished observation). There is no obvious metabolic function for the coupled regulation of these two enzymes; on the contrary, studies in vivo indicate that reciprocal regulation is the norm: in fasting rats, OTC is elevated and Glc-6-PD in the liver is reduced (Schimke, 1962; Glock & McLean, 1955). It seemed possible that any association between the expression of OTC and Glc-6-PD might reflect the chromosomal localization of the two structural genes. Both are X-linked, though it is not known if they map close together (Glc-6-PD is X-linked in the rat: Yoshida, 1978; OTC is known to be X-linked in man and mouse: Ricciuti, Gelehrter & Rosenberg, 1976; DeMars, LeVan, Trend & Russell, 1976). Although the cells in the present study are male in origin (Reuber, 1961), it nevertheless seemed possible that their failure to express two X-linked genes might well result from some process similar to X-inactivation. This would be expected to influence a set of syntenic genes regardless of the metabolic functions of their various products. It was with this in mind that special attention was given to the effects of 5-azacytidine on the expression of OTC and Glc-6-PD. Incorporation of 5-azacytosine into cellular DNA reduces the methylation of the DNA, and can activate the expression of previously silent genes. In particular, in certain circumstances, 5-azacytidine has been shown to re-activate genes on the inactive X-chromosome derived from a female cell (Mohondas, Sparkes & Shapiro, 1981; Lester, Korn & DeMars, 1982). Moreover, it seemed worthwhile to seek an effect of azacytidine on the hepatomas, in view of the increasing evidence that the methylation of DNA may play a general role in the control of gene expression. For reviews of this topic see Razin & Riggs (1980), Ehrlich & Wang (1981) and Bird (1984). Delers, Szpirer, Szpirer & Saggioro (1984) have recently independently discovered that 5-azacytidine increases OTC expression in Fu5 cells, in agreement with the conclusions reached below.
MATERIALS AND METHODS

Cells and culture media

Fu5 is a clonal derivative of the rat hepatoma H4-II-E (Pitot et al. 1964) and was kindly supplied to this laboratory by Dr M. C. Weiss. Fu5-TG1 was derived by selecting ultraviolet-mutagenized Fu5 cells in medium containing 6 μg/ml 6-thioguanine. The thioguanine resistance of Fu5-TG1 is not relevant to the experiments in this paper; it is probably significant that Fu5·TG1 is a relatively recent clonal isolate.

Cells were routinely maintained as monolayers in 25 or 75 cm² plastic tissue-culture flasks in 'arginine medium' containing 10% foetal calf serum. The media used in these experiments are described in detail elsewhere (Goss, 1984a). Arginine-medium is an enriched form of Eagle's Minimal Essential Medium, and 'ornithine-medium' is the same medium with 5 mM-ornithine in place of arginine. Unless otherwise specified, ornithine-medium was prepared with dialysed foetal calf serum (5%, v/v) and a supplement of 10 mM-N-carbamoylglutamate (GluNCbm). No hormonal supplements were used.

Selection, using diamide, for cells expressing glucose-6-phosphate dehydrogenase

This technique is essentially that of Rosenstraus & Chasin (1977). The cells were seeded into 25 cm² flasks (10⁴ cells/flask) in arginine-medium with 10% serum and left overnight to attach. Then diamide (1,1'-azobis(N,N-dimethylformamide): Aldrich Chemical Company, Gillingham, Dorset) was added to the medium to a concentration of 50 μM, and the cells were incubated in this medium at 37°C for 4 h. The treated cultures were rinsed well with phosphate-buffered saline, fed with fresh diamide-free medium, and returned to the incubator. Clones of surviving cells grew up within 2 weeks. The effectiveness of this selective procedure is described in Results.

Treatment of the cells with azacytidine

The cells to be treated were seeded into 75 cm² flasks in arginine-medium supplemented with 1% serum and 10 mM-GluNCbm. When they were growing well and about a quarter confluent, they were fed with similar medium containing 5-azacytidine (Sigma, St Louis, MO, U.S.A.), at the desired concentration, in place of the normal pyrimidine nucleotide supplement (see Goss, 1984a). Stocks of sterile azacytidine were prepared 100 times concentrated, and stored at -20°C for no more than 2 weeks before they were used. These stocks were thawed just before use, and any unused thawed solution was discarded. The cells were incubated in azacytidine-containing medium for up to 4 days, the medium being renewed daily. A graded toxicity was observed, according to the dose of azacytidine and the length of exposure. After this treatment the cultures were rinsed and allowed to recover, with sub-culturing as necessary, in arginine-medium with 1% serum, 10 mM-GluNCbm and the pyrimidine nucleoside supplement. At the end of the recovery period, the cells were harvested for enzyme assay, or transferred to selective medium. The use of medium with a low concentration of serum and with GluNCbm is not essential for these experiments, but it seemed a wise precaution in view of the evidence that such medium can assist the expression of urea-cycle enzymes in certain variant hepatomas (Goss, 1984b). This medium did seem to improve the reproducibility of the experiments.

Enzyme assays: preparation of sonicates

The cells were assayed when they reached confluency. Cell sonicates were prepared in 100 mM-maleic acid/sodium maleate buffer (pH 7.2) to give a protein concentration of 3–10 mg/ml. The protein concentrations were determined according to the method of Lowry, Rosebrough, Farr & Randall (1951), using bovine serum albumin standards.

Assay of ornithine transcarbamoylase (EC 2.1.3.3) and of carbamoylphosphate synthetase I (EC 2.7.2.5)

The assays for ornithine transcarbamoylase (OTC) and carbamoylphosphate synthetase I (CPS-I) are described in detail elsewhere (Goss 1984a). The limit of detection of OTC is around
0.1-0.2 nmol/min per mg, and duplicate assays on any one sonicate were generally found to agree within 10%. However, as has been noted before (Goss, 1984a), there is a much greater variation between assays on extracts of independent but supposedly identical cultures. In view of this, most sonicates were assayed only once, but then only the most obvious effects were taken into account when the results were interpreted.

Assay of glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and of 6-phosphogluconate dehydrogenase (EC 1.1.1.43).

A sample of cell sonicate (40 μl) was added to a buffered reaction mixture containing glucose 6-phosphate (Glc-6-P) or 6-phosphogluconate (6-P-GlcA), and nicotinamide adenine dinucleotide phosphate (NADP). The rate of reduction of NADP at room temperature was followed with a recording spectrophotometer at 340 nm. The reaction mixture consisted of: 0.9 ml 100 mM potassium dihydrogen phosphate, adjusted to pH 7.6 with 10 mM NaOH; 100 μl NADP solution (NADP disodium salt, 5 mM, in water: Boehringer Biochemicals, Lewes, Sussex); and 100 μl or Glc-6-P or 6-P-GlcA solution (Glc-6-P disodium salt, or 6-P-GlcA trisodium salt, both 50 mM, in water: Boehringer). The absorbance was recorded against a blank containing water in place of the Glc-6-P or 6-P-GlcA solution. Under these conditions, after an initial lag, the reaction rate was constant and proportional to the amount of protein assayed, to an absorbance of at least 0.2 o.D. unit. The lowest specific activity of either enzyme that could be detected reliably by this method was approximately 10 nmol/min per mg, and the results were highly reproducible (to within 10%).

Many samples contained both glucose-6-phosphate dehydrogenase (Glc-6-PD) and 6-phosphogluconate dehydrogenase (6-P-GlcAD). Since the product of Glc-6-PD is the substrate of 6-P-GlcD, it was possible that the Glc-6-PD assay might overestimate the true Glc-6-PD activity, because some 6-P-GlcAD activity would be recorded simultaneously. This was not a significant problem, as was shown by comparing the Glc-6-PD activity of a sample measured by the method described above with that measured as the increment in the rate of NADP reduction when Glc-6-P was introduced into an assay mixture that was already saturated with 6-P-GlcA. This result was verified in reconstruction experiments using purified enzymes obtained from Boehringer Biochemicals.

RESULTS

Spontaneous re-expression of the genes for OTC and Glc-6-PD in Fu5 and Fu5-TG1

Fu5 and Fu5-TG1 have only trace amounts of OTC and no detectable Glc-6-PD. When either cell line is plated in ornithine-medium, sub-clones arise that have greatly increased levels of OTC. Likewise, both cell lines are very susceptible to killing by diamide, and yet both yield some survivors after diamide treatment. In the case of Fu5, these survivors have been pooled and found to have high levels of Glc-6-PD (Table 1). There is some quantitative difference between the responses of Fu5 and Fu5-TG1 to the selective media: in particular, Fu5 yields many more diamide survivors than does Fu5-TG1. That Fu5 populations are rich in Glc-6-PD+ cells is also suggested by the high frequency with which Deschatrette & Weiss (1974) isolated Glc-6-PD+ clones from Fu5 without the assistance of diamide selection. This relatively high ‘contamination’ with Glc-6-PD+ cells is absent from Fu5-TG1, which is a recent clonal derivative of Fu5. Despite this purification by cloning, Fu5-TG1 readily gives rise to sub-clones able to grow in ornithine-medium: the re-expression of OTC must occur spontaneously with a fairly high frequency. It is not possible to give an accurate estimate of this frequency, as the number of clones surviving in ornithine-medium is somewhat variable, and not proportional to the number of cells seeded (a
Azacytidine-induced expression of OTC and Glc-6-PD

Table 1. Spontaneous re-expression of OTC and Glc-6-PD in Fu5 and Fu5-TG1

A. Cell survival in selective media

<table>
<thead>
<tr>
<th>Cells</th>
<th>Clones arising in ornithine</th>
<th>Clones surviving diamide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$10^6$ cells/flask</td>
<td>$10^5$ cells/flask</td>
</tr>
<tr>
<td>Fu5</td>
<td>$\bullet$140, $\bullet$200</td>
<td>4, 0</td>
</tr>
<tr>
<td>Fu5-TG1</td>
<td>Approx. 1000</td>
<td>300, 87</td>
</tr>
</tbody>
</table>

B. Specific activities of OTC and Glc-6-PD (nmol/min per mg)

<table>
<thead>
<tr>
<th>Cell</th>
<th>Before selection OTC/Glc-6-PD</th>
<th>Selected in ornithine OTC/Glc-6-PD</th>
<th>Selected in diamide OTC/Glc-6-PD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fu5</td>
<td>0-1/nil</td>
<td>15/n.a.</td>
<td>4/540</td>
</tr>
<tr>
<td>Fu5-TG1</td>
<td>0-4/nil</td>
<td>23(37)/20</td>
<td>n.a.</td>
</tr>
<tr>
<td>Fu5-TG1</td>
<td>0-2/nil</td>
<td>17/40</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

A. Samples of counted cells were either selected in ornithine (i.e. they were transferred to ornithine-medium with 10% dialysed serum and 1 mm-N-acetylglutamate), or else they were treated with diamide as described in Materials and Methods. All tests were done in 25 cm$^2$ flasks with 5 ml medium. Clones greater than 1 mm in diameter were scored at 2 weeks. Results are shown for two or more flasks in most instances. Both cell lines have a cloning efficiency of about 70% in standard arginine-medium.

• These figures are approximate, as these cultures showed a background of small clones in addition to the larger ones that were counted.

B. The hepatomas were assayed for OTC and Glc-6-PD before and after selection by ornithine-medium or by diamide. The clones surviving selection were pooled and grown to confluency for assay. Cells selected in ornithine-medium were assayed from that medium; though, if they were returned to arginine-medium, their OTC level remained elevated (result for Fu5-TG1 in parenthesis).

For Fu5-TG1 the results of two independent experiments are shown: variation between independent, equivalent cultures is greater than that between repeated assays on the same extract (see Materials and Methods). OTC results below 1 unit are rounded to the nearest 0-1 unit; higher OTC results are rounded to the nearest whole unit. Glc-6-PD results are rounded to the nearest 5 units; nil, <10 nmol/min per mg. n.a., not assayed.

The data are, however, clear enough to suggest that the reappearance of OTC does not require reversion of a genetic mutation. Nor is the reappearance of OTC attributable to a regulatory response to arginine withdrawal: the OTC level in Fu5-TG1 derivatives selected in ornithine-medium is stably maintained when they are transferred back to arginine-medium (Table 1).

Table 1 also shows that the selection of Glc-6-PD$^+$ cells with diamide produces a population of cells with slightly raised OTC levels. Likewise, selection in ornithine-medium is associated with a slight elevation of Glc-6-PD levels. This apparent association between the expression of the two genes is further investigated below in experiments studying the effects of azacytidine. Most of these experiments were done with Fu5-TG1 cells in order to take advantage of their very low initial level of diamide resistance.
Azacytidine-induced expression of OTC in Fu5-TG1

Following a suitable treatment with azacytidine, Fu5-TG1 cells can grow in ornithine-medium as a bulk culture with virtually no cell death; $10^6$ cells seeded in a 25 cm$^2$ flask can reach confluency within a week. This result has been obtained on

![Azacytidine treatment](image)

Fig. 1. Growth of Fu5-TG1 in ornithine medium: effect of azacytidine pretreatment. Fu5-TG1 cells were treated with azacytidine as indicated and, after a period of recovery (given in Table 2), $10^5$ cells were plated in ornithine-medium. When these tests were set up, the various batches of cells were also plated at low density in standard arginine-medium: in each case a plating efficiency of about 70% was obtained, regardless of azacytidine pretreatment. The cultures in ornithine-medium were fixed after 10 days, and stained with methyl violet for photography.
Azacytidine-induced expression of OTC and Glc-6-PD

three occasions: following a two-day treatment with 10 μM-azacytidine and 4 days of
recovery before testing, and following 3- and 4-day treatments with 9 μM-azacytidine
and 5 days of recovery. In the same experiments, when 10^5 (instead of 10^6) cells were
plated in ornithine-medium, there was considerable cell death and then clones grew
up; the results of a typical experiment are shown in Fig. 1. In contrast, when only
10^4 cells were plated there were no survivors. This marked dependence of plating
efficiency on cell density suggests that the cells do not uniformly regain the capacity
to synthesize arginine from ornithine, and that at high cell densities the cells best able
to synthesize arginine can assist the others by metabolic cooperation. Nevertheless,
the effect of azacytidine is quite clearly seen in Fig. 1. Cell survival in ornithine-
medium is greater the larger the dose and the longer the exposure time. Duplicates
of the flasks shown in Fig. 1 were grown up and assayed for OTC. The control culture
(no azacytidine pre-treatment) and the culture with the strongest azacytidine pre-
treatment (9 μM for 4 days) were found to have similar specific activities of OTC, 17
and 20 nmol/min per mg, respectively, showing that the OTC level finally attained
is independent of azacytidine pre-treatment. It is likely that the OTC activity seen in
azacytidine-treated cultures results from the induction in the cells of changes similar
to those that would otherwise occur spontaneously, albeit much more rarely.

In the experiments described above, the cells were assayed for OTC (and for Glc-
6-PD: see below) at the time of their plating into test media. The results, given in
Table 2, show that the longer treatments with azacytidine produce an elevation of
OTC levels that is detectable even before the cells are subjected to selection. It is not,
however, possible to discern a clear relationship between the OTC levels and the dose
of azacytidine, but this is hardly surprising in view of the variation in OTC levels
commonly seen in duplicate cultures (see Materials and Methods).

The experiments discussed so far are the most recent in a long series. Earlier
experiments were done under a wide range of conditions, and gave results of varying

<table>
<thead>
<tr>
<th>Azacytidine treatment</th>
<th>Enzyme activities (nmol/min per mg)</th>
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<tbody>
<tr>
<td>Dose (μM)</td>
<td>Exposure (days)</td>
</tr>
<tr>
<td>-----------------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>Experiment 1</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
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<tr>
<td>9</td>
<td>4</td>
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<tr>
<td>Experiment 2</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>9</td>
<td>4</td>
</tr>
</tbody>
</table>

Fu5-TG1 cells were treated with azacytidine as described in Materials and Methods. The enzyme
assays were done after a period of recovery, when the cells had reached confluence. Two experiments
are shown: cells from the first experiment were used, at the time of assay, to set up growth tests in
ornithine-medium (shown in Fig. 1), and to set up diamide-survival tests (shown in Table 3). Enzyme
activities are expressed as in Table 1.
Table 3. Selection of Fu5-TG1 with diamide: the effects of azacytidine pretreatment and of selection in ornithine-medium

<table>
<thead>
<tr>
<th>Azacytidine treatment</th>
<th>Dose (µM)</th>
<th>Exposure (days)</th>
<th>Recovery (days)</th>
<th>Selected ornithine-medium</th>
<th>Clones arising after diamide treatment (10⁴ cells seeded)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>5, 5</td>
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<td>3</td>
<td>2</td>
<td>7</td>
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<td>7</td>
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<td>7, 28</td>
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<td></td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>-</td>
<td>13, 21</td>
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<td>43, 80</td>
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<td>9</td>
<td>4</td>
<td>5</td>
<td>+</td>
<td>238, 299, 211</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
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<tr>
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<td>0</td>
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<td>0</td>
<td>-</td>
<td>0, 0</td>
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<tr>
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<td>3</td>
<td>3</td>
<td>5</td>
<td>-</td>
<td>2, 8</td>
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<tr>
<td></td>
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<td>0</td>
<td>+</td>
<td>327</td>
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<tr>
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<td>3</td>
<td>3</td>
<td>5</td>
<td>+</td>
<td>403</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>3</td>
<td>5</td>
<td>+</td>
<td>241</td>
</tr>
</tbody>
</table>

Fu5-TG1 cells were tested by the standard method for resistance to killing by diamide (see Materials and Methods). In most instances the tests were set up in duplicate. Various azacytidine pretreatments were tried, as indicated, and, in some cases, the cells were selected in ornithine-medium before being challenged with diamide. Two experiments are shown: the first is the same experiment as that shown in Fig. 1 and in Table 2 (experiment 1 of Table 2).

clarity. It is, however, worth noting that azacytidine-induced re-expression of OTC was seen in nine out of twelve independent experiments, and that two of the three failures can be attributed to the use of two little azacytidine and too short an exposure-time. The action of azacytidine is apparently highly repeatable.

Azacytidine-induced expression of Glc-6-PD in Fu5-TG1

Table 2 shows that Glc-6-PD levels are increased in Fu5-TG1 after azacytidine treatment. As with OTC, the longer treatments with azacytidine are more effective. Furthermore, there is some indication that the increase of Glc-6-PD is related to the dose of azacytidine. These observations are supported by the results of diamide selection on azacytidine-treated cells: the greater the dose, and the longer the treatment with azacytidine, the more cells survive exposure to diamide (Table 3). Now diamide has a slight toxicity even for Glc-6-PD+ cells: in a control experiment, some diamide-selected cells were subjected to a second diamide treatment, and their plating efficiency was subsequently found to be 25%. This level of toxicity agrees well with that shown by Rosenstraus & Chasin (1977). It follows that the results in Table 3 significantly underestimate the extent of Glc-6-PD expression induced by azacytidine.

The action of azacytidine on Fu5 cells

Only one experiment with azacytidine has been done on Fu5: the cells were treated
Azacytidine-induced expression of OTC and Glc-6-PD

with 9 μM-azacytidine for 4 days, and then tested after 5 days of recovery; 10^5 cells were seeded in ornithine-medium in each of two 25 cm^2 flasks. Both flasks developed approximately 300 clones, whereas two control flasks set up with untreated cells yielded just one clone and 19 clones, respectively. Further, similar control data are to be found in Table 1. In the same experiment, the cells were also tested for resistance to diamide: 260 clones survived from 10^4 azacytidine-treated cells, as compared to 218 from 10^4 control cells. It appears that azacytidine has an obvious effect in increasing the survival of Fu5 in ornithine-medium, but that much more work is needed to establish if there is any effect of azacytidine on diamide resistance in this cell line.

Further evidence that OTC and Glc-6-PD tend to be re-expressed together

On two occasions, when Fu5-TG1 cells had been tested for growth in ornithine-medium and for diamide resistance, the clones that grew up in ornithine-medium were pooled, and then they too were tested with diamide. The results, given in Table 3, show that selection in ornithine-medium produces a population of cells with significantly increased resistance to diamide. This constitutes further evidence of an association between re-expression of OTC and Glc-6-PD. In the absence of azacytidine pretreatment, this association could be demonstrated directly by enzyme assay (Table 1); however, if the cells had first been treated with azacytidine, so that their Glc-6-PD was already slightly raised, it was not possible to detect any obvious further increase in Glc-6-PD when the cells were subsequently selected in ornithine-medium (data not shown). Of course, even a considerable increase in diamide resistance requires that only a minority of cells have increased levels of Glc-6-PD. The resulting increment in the overall level of Glc-6-PD could well be small and difficult to demonstrate, except in cells that initially have no detectable enzyme at all. To avoid this technical problem, it would be necessary to assay Glc-6-PD on clones of cells isolated before and after selection in ornithine-medium. Such a clonal analysis is described below, but there it is used to investigate the elevation of OTC levels that occurs when cells are selected for Glc-6-PD (i.e. the complementary effect to the one described in this section).

Clonal analysis of OTC and Glc-6-PD

In the experiments described above, OTC and Glc-6-PD were assayed in uncloned populations of cells. This leads to significant ambiguities: for example, a partial recovery of enzyme activity could be due either to the majority of cells expressing a low level of enzyme, or to a full recovery of enzyme activity in a small minority of cells. A clonal analysis should resolve this problem, and reveal whether, in this system, gene expression is infinitely variable, or whether it obeys an all-or-none law. Furthermore, such an analysis should be informative regarding the nature of the association of re-expression of OTC and Glc-6-PD. Three sets of sub-clones were prepared from Fu5-TG1: a control set (set C) from untreated cells, a set from azacytidine-treated cells (set A), and a set derived by selecting the azacytidine-treated cells with diamide (set D). The results of OTC and Glc-6-PD assays on these clones are given in Table 4 and in Fig. 2.
Table 4. Specific activities of OTC and Glc-6-PD in clones isolated from Fu5-TG1

<table>
<thead>
<tr>
<th>Specific activities (nmol/min per mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clone</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Clone</th>
<th>OTC/Glc-6-PD</th>
<th>OTC/Glc-6-PD</th>
<th>OTC/Glc-6-PD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.5/nil</td>
<td>4.3/35</td>
<td>153/355</td>
</tr>
<tr>
<td>2</td>
<td>0.7/nil</td>
<td>1.3/nil</td>
<td>6/365</td>
</tr>
<tr>
<td>3</td>
<td>0.6/nil</td>
<td>1.7/nil</td>
<td>4.720</td>
</tr>
<tr>
<td>4</td>
<td>1.0/nil</td>
<td>1.7/nil</td>
<td>145/420</td>
</tr>
<tr>
<td>5</td>
<td>1.2/nil</td>
<td>2.1/nil</td>
<td>17/425</td>
</tr>
<tr>
<td>6</td>
<td>3.4/nil</td>
<td>2.3/30</td>
<td>3.505</td>
</tr>
<tr>
<td>7</td>
<td>1.8/nil</td>
<td>4.8/370</td>
<td>93/600</td>
</tr>
<tr>
<td>8</td>
<td>1.5/nil</td>
<td>11.3/nil</td>
<td>78/215</td>
</tr>
<tr>
<td>9</td>
<td>0.7/nil</td>
<td>2.3/nil</td>
<td>12/480</td>
</tr>
<tr>
<td>10</td>
<td>2.0/nil</td>
<td>1.2/nil</td>
<td>296/960</td>
</tr>
<tr>
<td>11</td>
<td>1.1/nil</td>
<td>2.6/nil</td>
<td>141/190</td>
</tr>
<tr>
<td>12</td>
<td>2.2/nil</td>
<td>7.3/nil</td>
<td>54/420</td>
</tr>
<tr>
<td>13</td>
<td>2.8/nil</td>
<td>13.1/nil</td>
<td>66/460</td>
</tr>
<tr>
<td>14</td>
<td>1.2/nil</td>
<td>3.0/nil</td>
<td>9/600</td>
</tr>
<tr>
<td>15</td>
<td>4.6/nil</td>
<td>nil/nil</td>
<td>66/460</td>
</tr>
<tr>
<td>16</td>
<td>1.0/nil</td>
<td>nil/nil</td>
<td>9/600</td>
</tr>
<tr>
<td>17</td>
<td>0.7/nil</td>
<td>8.0/nil</td>
<td>6/385</td>
</tr>
<tr>
<td>18</td>
<td>1.1/nil</td>
<td>23.5/nil</td>
<td>31/1260</td>
</tr>
<tr>
<td>19</td>
<td>0.4/nil</td>
<td>1.8/nil</td>
<td>4/420</td>
</tr>
<tr>
<td>20</td>
<td>0.5/nil</td>
<td>16.3/nil</td>
<td>289/nil</td>
</tr>
</tbody>
</table>

Sub-clones of Fu5-TG1 were isolated, grown to confluency in 25 cm² flasks in arginine-medium (with 10% serum), and then assayed for OTC and Glc-6-PD. Set C clones were isolated from untreated cells. Set A clones were isolated from cells that had been given 4 days of treatment in 9 μg-azacytidine and then allowed to recover for 5 days before cloning. These azacytidine-treated cells were also used to prepare set D. Set D clones arose after azacytidine-treated cells had been exposed to diamide. The standard diamide treatment (see Materials and Methods) was applied to 10⁴ cells in a 75 cm² flask. Glc-6-PD results are rounded to the nearest 5 units; nil, not detectable (i.e. <10 nmol/min per mg). OTC results are rounded to the nearest 0.1 unit (sets C and A), or to the nearest unit (set D): nil, not detectable (i.e. <0.1 nmol/min per mg).

The control clones (set C) have low levels of OTC and, with one exception, their Glc-6-PD was undetectable. (The one Glc-6-PD+ clone has a very low specific activity of that enzyme.) The OTC levels in these clones were found to be slightly higher (averaging 1.45 nmol/min per mg) than had been expected from assays on uncloned Fu5-TG1 cells (typically 0.4 nmol/min per mg or less). The significance of this small difference has not been investigated.

The clones isolated after azacytidine treatment (set A) show a general upward shift in OTC activity in comparison to the control clones: out of 18 treated clones, all but two had specific activities of OTC greater than 1.2 nmol/min per mg, whereas, out of 20 control clones, 13 had less OTC than this (Fig. 2). Furthermore, seven of the treated clones had OTC levels greater than 4.6 nmol/min per mg, the highest level recorded in the control clones. It appears that, when Fu5-TG1 cells are treated with
Fig. 2. Specific activities of OTC in clones of Fu-TG1. This is a graphic representation of the OTC assay results listed in Table 4. Each result is plotted as a bar to facilitate comparison of the assays on the different sets of clones: C, clones of control Fu5-TG1; A, clones of azacytidine-treated Fu5-TG1; D, diamide-resistant clones selected from azacytidine-treated Fu5-TG1. For details of the treatments, see Table 4. Where the results are too closely clustered to be resolved in this figure, they are indicated by a block labelled with the number of results included.
azacytidine, most cells show some increase in OTC expression. A corresponding general increase in Glc-6-PD could not be demonstrated, but this could merely reflect the lesser sensitivity of the Glc-6-PD assay. However, one azacytidine-treated clone, A7, did show a high specific activity of Glc-6-PD. Since, in control populations of Fu5-TG1, cells resistant to diamide occur very rarely, it is likely that this clone represents an instance of azacytidine-induced re-expression of Glc-6-PD. A sample of the azacytidine-treated cells that were used to prepare the set A clones was tested with diamide: $10^4$ cells yielded over 400 surviving colonies, so it is not surprising that a clone with high Glc-6-PD levels should have been detected among the 18 clones in set A.

As expected, the clones surviving diamide (set D) show high levels of Glc-6-PD. Just one exceptional clone lacking Glc-6-PD was detected: apparently diamide selection is slightly leaky (or, alternatively, Glc-6-PD expression may sometimes be transient). The diamide survivors were clearly enriched with clones expressing high levels of OTC: half the clones of set D have OTC levels much higher than the highest level detected in set A (Fig. 2). The range of OTC in the diamide-selected clones is, however, very great (3–296 nmol/min per mg), so it is unlikely that the diamide treatment itself either causes or selects directly for an elevation of OTC. (There is, of course, no expectation that diamide could have such a direct effect on OTC expression.) Rather, it would appear that there is a tendency for ‘full’ recovery of both Glc-6-PD and OTC to occur in the same cells. The clonal analysis shows that this association of effects is not obligatory: some clones have a high level of Glc-6-PD and a low level of OTC, and, in other clones, OTC is elevated in the absence of Glc-6-PD (Table 4). The levels of OTC and Glc-6-PD are therefore being regulated by a mechanism the action of which can be confined so as to affect either enzyme independently of the other. Further consideration of the consequent distribution of enzyme activities is included in the Discussion.

The three sets of clones were subjected to other tests the results of which are not shown in detail, but do deserve brief comment. At least half the clones in each set were assayed for CPS-I. This revealed no significant variation between the sets, so there is no reason to suppose that the variation in OTC levels is due to some fundamental control of the expression of tissue-specific traits in general, or of urea-cycle enzymes in particular. In the same vein, several clones representing the extremes of Glc-6-PD expression were also assayed for 6-phosphogluconate dehydrogenase. The activities of these two enzymes have been shown to change coordinately in vivo, as changes in dietary state vary the requirement for the hexose monophosphate shunt (Fitch & Chaikoff, 1960). However, 6-P-GlcAD was found to be present at about the same specific activity in all the clones, whether their Glc-6-PD level was high or low. The same clones were then challenged in a series of tests with insulin, dibutyryl cyclic adenosine monophosphate, dexamethasone and 3,3',5-triiodo-l-thyronine. A number of studies suggested that these factors might regulate the levels of Glc-6-PD and 6-P-GlcAD (Rudack, Chisholm & Holten, 1971a; Rudack, Davie & Holten, 1971b; Huggins & Yao, 1959; Melsicek & Towle, 1982). However, in no case was there any obvious alteration in the activity of either enzyme. It was thus not possible to establish
any connection between the variation of Glc-6-PD expression in these clones and the normal regulation of the hexose monophosphate shunt.

DISCUSSION

The suppression of OTC and Glc-6-PD in FuS-TGl resembles X-inactivation.

It will be clear from the experiments described above that, in Fu5-TG1 cells, the activities of OTC and Glc-6-PD are subject to regulation by similar or identical mechanisms. (Fu5 cells appear to show the same phenomenon, but they have not been investigated so fully.) In untreated cultures, OTC is barely detectable and Glc-6-PD is not detectable, yet either enzyme may be regained spontaneously, and the re-appearance of both can be induced by azacytidine treatment. Once the enzymes are regained, their presence is stably maintained without the need for any continued inducing stimulus. In view of the known effects of azacytidine, it is obvious to suggest that DNA methylation is the controlling factor. Most simply, in Fu5-TG1, the structural genes for OTC and Glc-6-PD could be suppressed by methylation, and derivatives of Fu5-TG1 re-expressing these genes could then arise by a spontaneous or azacytidine-induced reduction in that methylation. This model is identical to that proposed by Mohondas et al. (1981) and Lester et al. (1982) to account for the maintenance of natural X-inactivation, and for its reversal by azacytidine. Since frequent reference is made below to the work of these authors, it is convenient here to give a brief description of their experimental approach. They investigated the reactivation of three human genes (those for hypoxanthine phosphoribosyl transferase, HPRT; phosphoglycerate kinase, PGK; and Glc-6-PD) that were present on a Lyonized X-chromosome contained in some mouse–human hybrid cells. These cells lacked murine HPRT, but expressed the murine forms of both PGK and Glc-6-PD. In this situation, the appearance of human HPRT could be detected by the use of HAT selective medium (Littlefield, 1964), but electrophoretic enzyme separations were necessary to detect the appearance of human PGK and Glc-6-PD. The rat hepatomas used in the present work are genetically less well-characterized than these hybrid cells, but the hepatomas nevertheless provide a useful system for investigating azacytidine-induced gene expression: they contain two suitably suppressed genes whose activation can, in both cases, be detected by the use of selective media, and, furthermore, the activities of both these genes can be assessed directly by quantitative enzyme assay.

OTC and Glc-6-PD tend to be re-expressed coordinately.

It has been shown above that either OTC or Glc-6-PD can be regained alone. Occasionally, they might be expected to reappear together. However, since cells selected for either enzyme are markedly enriched in cells that have also regained the other enzyme, such coordinate re-expression cannot be explained simply by chance. These findings are easily accommodated by the suggestion that the reappearance of the enzymes requires a reduction in the methylation of their structural genes. DNA methylation would be expected to act primarily locally, so the two genes could be
controlled independently. The tendency of both genes to be reactivated in the same cells might be explained, if the genes are sited close together, by suggesting that sufficient demethylation could so alter the conformation of the chromatin that a group of genes could be affected simultaneously. There is, of course, in position-effect variegation, ample precedent for the variable propagation of a controlling influence within a segment of chromosome (Baker, 1968; Cattanach & Isaacson, 1967). Alternatively, a more trivial explanation for the simultaneous reappearance of both enzymes is that some cells might incorporate more azacytidine than others. However, it must be remembered that the reactivation of OTC and Glc-6-PD is non-random even in the absence of azacytidine treatment. Both Mohondas et al. (1981) and Lester et al. (1982) have looked for non-randomness in gene reactivation. Mohondas et al. observed that clones selected for the re-expression of HPRT frequently also re-expressed PGK or Glc-6-PD, but, unfortunately, these authors did not determine the frequency of re-expression of PGK and Glc-6-PD in clones not selected for HPRT. Lester et al. made a careful study of the re-expression of HPRT and PGK, and concluded that these two genes behaved independently. It is then apparent that the behaviour of PGK and HPRT in the hybrid cell system is significantly different from that of OTC and Glc-6-PD in our hepatoma system. Lester et al. stress that PGK and HPRT are far apart on the X-chromosome: it could be that such a wide separation is required if genes are to respond independently to changes in DNA methylation.

The effectiveness of azacytidine in activating gene expression

Several points arise from the clonal analysis of OTC and Glc-6-PD activities. Let us first consider the OTC results. Apparently, the majority of cells treated with azacytidine subsequently showed some elevation of OTC. That azacytidine can be so effective is known from other work: this point is made especially clearly by Harris (1982). The clonal analysis also showed that a very wide range of OTC levels can result from azacytidine treatment. Lester et al. (1982) found a similar effect. They accepted that, in their experiments, some of the variation in enzyme levels could have been due to trivial genetic differences between their various hybrid cells, but they believed that the major cause was likely to be fundamental to the process of gene reactivation itself; for instance, there could be variations in the degree or pattern of demethylation of the DNA. This view is supported by the present work, where widely varying enzyme levels were seen in cells that are likely to be genetically less heterogeneous than mouse–human hybrids. The hepatomas showed such a broad continuum of OTC levels that it was not possible sensibly to divide the clones into OTC+ and OTC− groups. There will, of course, be some threshold of enzyme activity that is required for cell growth in ornithine-medium, but the definition of that threshold is complicated considerably by metabolic cooperation. For instance, clone A7, with an OTC activity of about 5 nmol/min per mg, was able to grow well, with no cell death, when 5 × 10^5 cells were seeded into 5 ml ornithine-medium in a 25 cm^2 flask. However, a corresponding inoculum of 10^3 cells all died, without even giving rise to any clones. The use of ornithine-medium to detect OTC expression is convenient, in that very large numbers of cells may be screened rapidly, but it has to
Azacytidine-induced expression of OTC and Glc-6-PD

be accepted that this approach will underestimate the true effectiveness of azacytidine. It is interesting in this context to note that Lester et al. (1982) concluded that azacytidine induced the re-expression of PGK much more often than that of HPRT. Of course there is no reason to suppose that all genes should be equally responsive to azacytidine, but, nevertheless, the conclusion of Lester et al. is difficult to assess because, as has been described above, different detection methods were used for the two enzymes: PGK was detected by electrophoretic enzyme assay, but HPRT was detected using a selective system.

Another aspect of OTC expression to emerge from our clonal analysis is the apparent clustering of the higher OTC levels in a geometric progression. This can be seen in Fig. 2, which shows groups of clones with OTC activities around 75, 150 and 300 nmol/min per mg. Peterson (1974) has described a similar progression in the rates of albumin synthesis in subclones of Fu5, and has shown that such a discontinuous distribution of genetic activity is a widespread phenomenon in mammalian cells. This observation is as yet unexplained, though the answer might well lie in the existence of multiple gene copies (Peterson, 1976).

If we now consider the results of the Glc-6-PD assays, it will be seen that they are distributed differently from the OTC results. With only a few exceptions, the clones show either a high level of Glc-6-PD, or none at all. It is quite possible, though, that this apparently all-or-none control of gene expression could be a technical artifact. The Glc-6-PD− group could result from the poor sensitivity of the Glc-6-PD assay, and the use of diamide selection is very likely to have biased the results at the other extreme, by excluding clones with intermediate levels of enzyme. Again, it is useful to consider the results of Lester et al. (1982). Since, in the hybrid cell system, Glc-6-PD had to be analysed by electrophoresis, accurate quantitative assays of human Glc-6-PD were not attempted. Nevertheless, these authors describe their electrophoretograms in considerable detail, and it would seem that they found a broader and more continuous range of Glc-6-PD expression than we did. In contrast, their quantitative assays of HPRT are less scattered, more like our Glc-6-PD results. Since Lester et al. selected for HPRT but not for Glc-6-PD these results are consistent with the view that the use of selective systems will reduce the range of gene expression that is detected.

The suppression of OTC and Glc-6-PD in Fu5-TG1 is apparently quite abnormal. It bears no detectable relationship to the natural regulation of the urea-cycle or the hexose monophosphate shunt. Instead, in many respects it resembles X-inactivation. Both X-inactivation, and the suppression of OTC and Glc-6-PD described here, are highly susceptible to reversal by azacytidine. Furthermore, in both cases, azacytidine treatment results in a wide range of levels of gene expression.

A novel aspect of the regulation of OTC and Glc-6-PD is that they tend to become reactivated coordinately. The cause of this is unknown, though it seems possible that genes could well behave in this way if they were situated close to each other on the chromosome. Then the reactivation of both genes might be associated with euchromatinization of a single block of heterochromatin, a possibility that is currently under investigation in this laboratory.
It remains to be established whether the suppression of gene activity by DNA methylation is a common cause of epigenetic variation in hepatomas. Hepatomas in vitro frequently develop stable changes of phenotype: typically, they lose the expression of some of their liver-specific traits (Deschatrette & Weiss, 1974). This variation is apparently non-mutational in origin (Deschatrette, Moore, Dubois & Weiss, 1980; Goss, 1984a), and, in the case of a suppressed albumin gene, an associated increase in DNA methylation has been reported (Ott et al. 1982). It will be of interest to see if variants of this type show any response to azacytidine treatment.

The author thanks Mrs Heather Smith for her excellent technical assistance, and also Professor H. Harris and the staff of the Dunn School for providing the support and encouragement necessary for this work. The work was financed by the Cancer Research Campaign.

Note added in proof Recent linkage studies of Glc-6-PD have shown that, in the mouse, both Glc-6-PD and OTC genes are situated in the proximal third of the X-chromosome. (Personal communication: Dr Jo Peters, MRC Radiobiology Unit, Harwell, U.K.)

REFERENCES


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(Received 14 May 1984 – Accepted 9 July 1984)