Expression of a human gene for polyamine transport in Chinese-hamster ovary cells

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A molecular-genetic approach towards isolating mammalian polyamine-transport genes and their encoded proteins was devised involving the production of Chinese-hamster ovary (CHO) cells expressing a human polyamine-transport protein. CHO cells and a polyamine-transport-deficient CHO mutant cell line (CHOMG) were equally sensitive to the antiproliferative effects of α -difluoromethylornithine (DFMO), which blocked endogenous polyamine synthesis. Exposure to exogenous polyamines increased intracellular polyamine levels and reversed this DFMO-induced cytostasis in the CHO cells, but not in the CHOMG cells. CHOMG cells were therefore transfected with human DNA (isolated from HT-29 colon carcinoma cells) and cells expressing the human polyamine-transport system were identified by the ability of these cells to grow in a medium containing DFMO and polyamines. A number of different positive clones were identified and shown to have the capacity for polyamine uptake and an increased sensitivity to the toxic effects of the polyamine analogue methylglyoxal bis(guanylhydrazone). Differences in these properties between the clones are consistent with a multiplicity of polyamine-transport systems. Some clones also showed a change in growth characteristics, which may indicate a relationship between genes involved in the polyamine-transport system and in cell proliferation.

INTRODUCTION

Although the enzymic capacity to synthesize polyamines appears to be a ubiquitous property of mammalian cells, it is well known that these cells also contain an uptake system capable of accumulating exogenous polyamines (Pegg & McCann, 1982; Jänne *et al.*, 1983; Tabor & Tabor, 1984; Pegg, 1986, 1988; Porter & Jänne, 1987). This system is energy-dependent, capable of concentrating polyamines and analogues against a substantial concentration gradient, regulated by hormones and growth factors, and responsive to changes in the intracellular polyamine concentration (Pohjanpelto, 1976; DiPasquale *et al.*, 1978; Alhonen-Hongisto *et al.*, 1980; Seppänen *et al.*, 1981; Jänne *et al.*, 1983; Rinehart & Chen, 1984; Feige & Chambaz, 1985; Feige *et al.*, 1986; Kakinuma *et al.*, 1988; Gawel-Thompson & Greene, 1988; Heston & Charles, 1988). These observations suggest that the system may play an important role in maintaining cellular polyamines at an optimal level for cell growth and that changes in the uptake system may be factors in the regulation of cellular polyamine levels.

It is also known that a number of polyamine analogues and related compounds, including methylglyoxal bis(guanylhydrazone) (MGBG) (Mandel & Flintoff, 1978; Alhonen-Hongisto *et al.*, 1980; Seppänen *et al.*, 1981; Jänne *et al.*, 1983; Rodrigues *et al.*, 1987), paraquat (Smith & Wyatt, 1981; Gordonsmith *et al.*, 1985; Byers

et al., 1987) and various alkylated spermidine and spermine derivatives (Porter *et al.*, 1984, 1987; Porter & Bergeron, 1988) may be substrates for the polyamine-transport system. These compounds are toxic to cells which take them up, and the activity of the polyamine transport system therefore contributes significantly to the sensitivity of mammalian cells towards these agents. Both MGBG and N^1N^{12} -bis(ethyl)spermine, which are taken up by the polyamine-transport system, have potential value as cancer chemotherapeutic agents. Their efficacy thus depends on the continued activity of this system.

In contrast, it appears that the ability to take up exogenous polyamines may counteract the antitumour properties of drugs such as α -difluoromethylornithine (DFMO), which exert their antiproliferative effects by blocking the endogenous synthesis of polyamines. Tumour cells grown *in vitro* in the presence of DFMO respond by a large increase in the activity of the polyamine-transport system (Alhonen-Hongisto *et al.*, 1980; Seppänen *et al.*, 1981; Jänne *et al.*, 1983), but in the absence of a supply of exogenous polyamines, this drug exerts a powerful inhibitory effect on the growth of such cells (Pegg & McCann, 1982; Jänne *et al.*, 1983; Porter & Jänne, 1987; Pegg, 1988). However, its effects on tumour growth *in vivo* have been much less impressive, and it is possible that the uptake of exogenous polyamines provided by the diet or from cell turnover may limit its action. Recent support for this concept has been provided

Abbreviations used: CHO, Chinese-hamster ovary; CHOMG, polyamine-transport-deficient, MGBG-resistant, mutant CHO-cell line; DFMO, α -difluoromethylornithine; MGBG, methylglyoxal bis(guanylhydrazone); FBS, fetal-bovine serum; MEM, minimal essential medium; PBS, phosphate-buffered saline (0.012 M- Na_2HPO_4 /0.0018 M- KH_2PO_4 /0.12 M- NaCl , pH 7.4); ODC, ornithine decarboxylase.

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by the demonstration that DFMO is much more active in mice carrying L1210 tumour cells if these cells are deficient in polyamine transport (Persson *et al.*, 1988).

It is clear that a more detailed characterization of the polyamine-transport system and a better understanding of its regulation and physiological importance is needed. At present, no proteins involved in the transport have been purified and studied in reconstituted systems, and such purification is extremely difficult, because of the small amount of material present. However, mutant cells lacking the transport capacity have been isolated (Mandel & Flintoff, 1978; Rodrigues *et al.*, 1987; Persson *et al.*, 1988; Heaton & Flintoff, 1988). In the present work we have made use of one of these mutants, and the observation that the mutant cells differ from cells expressing the transport system in their inability to grow in the presence of DFMO and exogenous polyamines, to isolate clones of CHO cells expressing a human gene involved in polyamine transport after transfection of human DNA into the mutant CHO cells. The properties of these clones, which are described here, are consistent with our previous suggestion (Byers *et al.*, 1987) that there may be heterogeneity in polyamine transporters. The clones should prove useful in the isolation of the human gene.

MATERIALS AND METHODS

Materials

The pSV₂-neo plasmid was generously provided by Dr. J. J. McCormick, Carcinogenesis Laboratory, Michigan State University, East Lansing, MI, U.S.A. MGBG was obtained from the Aldrich Chemical Co., Milwaukee, WI, U.S.A. DFMO was kindly donated by Merrell Dow Research Institute, Cincinnati, OH, U.S.A. The [*guanyldiazotization*-¹⁴C]MGBG [1,4-¹⁴C]putrescine, and [1,4-¹⁴C]spermine were purchased from Amersham-Searle.

Cell culture

The CHO cells were generously provided by Dr. W. Flintoff, Department of Microbiology, University of Western Ontario, London, Ontario, Canada. They were a MGBG-resistant line (CHOMG) and the parent CHO (Pro⁻) line. The cells were grown as monolayers in α -MEM (Gibco) with 10% (v/v) fetal-bovine serum (FBS), 36 mM-NaHCO₃, penicillin (12 μ g/ml) and streptomycin (12 μ g/ml) at 37 °C in 10% CO₂ and 93% humidity. These cells were harvested utilizing a 0.25% (w/v) buffered trypsin solution.

The HT-29 cells were grown as monolayers in Dulbecco's MEM containing 36 mM-NaHCO₃, gentamicin (50 μ g/ml)/3% glutamine/10% PBS at 37 °C in 10% CO₂ and 93% humidity. They were harvested by rinsing with 0.02% (w/v) EDTA in FBS before the addition of a 0.25% (w/v) buffered solution of trypsin.

Effect of polyamines on growth of cells treated with DFMO

The ability of exogenous polyamines to rescue CHO and CHOMG cells from DFMO-induced cytostasis was quantified as follows. Cells were grown in the presence and absence of 5.0 mM-DFMO for 72 h, after which the medium was aspirated off representative dishes and the cells trypsin-treated. Trypsin treatment was stopped by adding an equal volume of medium containing 10% FBS. The surface was gently scraped with a rubber

policeman to dislodge the cells and soft pipetting was used to break up any cell clumps before counting the cells on a haemocytometer or electronic cell counter (Coulter Electronics). The medium in the remaining dishes containing DFMO-treated cells was aspirated off and replaced with fresh medium containing 5.0 mM-DFMO/2.0 mM-aminoguanidine and, where indicated, different polyamines at various concentrations. At 72 h after the medium was changed, these cells were counted. The effect of exogenous polyamines on DFMO-induced cytostasis was expressed as the percentage of polyamine-treated cells maintained in DFMO relative to the number of DFMO-treated cells at 144 h. Aminoguanidine was added to prevent the oxidation of exogenous polyamines by bovine serum amine oxidases (Pegg *et al.*, 1981).

Effect of MGBG on cell growth

In these experiments, the initial cell number plated was such that the control cells would not reach confluence before the detrimental effect of the drug was determined. After the indicated length of exposure, the cell number was determined as described above.

Determination of neomycin resistance

Qualitative observations of cells exposed to G-418 (a neomycin analogue; 800 μ g/ml) were used to determine neomycin resistance. These cells were plated at 3×10^4 cells/60 mm dish in the presence of this drug and the observations were taken over a period of 7 days, which was a sufficient period for exposure to result in the death of negative control cells.

Proline auxotrophy

Cells to be tested for proline auxotrophy were weaned on to Dulbecco's MEM, which lacks proline, as follows. Cells grown in α -MEM, which contains 350 μ M-proline, were passaged into an intermediate medium (50% α -MEM/50% Dulbecco's MEM/350 μ M-proline) and then into Dulbecco's MEM supplemented with 350 μ M-proline. The cells were then washed three times with PBS at 37 °C, harvested, and plated with Dulbecco's MEM into 60 mm dishes with and without 350 μ M-proline. At 72 h, the numbers of cells from both groups were counted to determine the effect of proline starvation. At this time fresh media with and without proline was added to representative dishes containing starved cells. These dishes were harvested and the cell number counted at 144 h.

Cell growth

Cell growth curves were obtained by counting cells at various times after plating in 60 mm dishes. The time points were arranged such that growth was monitored in both the exponential and confluent stages of cell growth.

Selection of polyamine-transport-competent cells

The ability of DFMO and exogenous polyamines to select for polyamine-transport-competent cells was tested in three experiments. In each case, the cells were plated by a technician such that the pattern or location of the two cell types was unknown to the person making the reported observations. In the first experiment, CHO and CHOMG cells were plated into separate wells of a 24-well plate at 1×10^4 cells/well in the presence of 5.0 mM-DFMO, 2 mM-aminoguanidine and 5 μ M-putrescine. Cell growth and morphology were used as the criteria for

determining the cell line contained in each well. In the second experiment, freshly harvested CHO and CHOMG cells were diluted and a volume theoretically containing 1 cell was added to each well of a 96-well plate. After 3 days, at which point single colonies were evident, the medium was changed to contain 5.0 mM-DFMO, 2 mM-aminoguanidine and 5 μ M-putrescine. Differential responses of CHO and CHOMG cells to this selective medium were used to score wells. Plating resulted in 59 colonies, each in a separate well. On the basis of cell morphology and growth, 22 colonies were correctly identified as CHOMG cells, and the remaining 37 colonies were properly classified as CHO cells. In the third experiment, CHO cells were diluted and volumes containing either 1 or 10 cells was added to five dishes each containing 3×10^4 CHOMG cells in the presence of 5 mM-DFMO. After 3 days the medium was changed to fresh medium (5 mM-DFMO/2 mM-aminoguanidine/5 μ M-putrescine). The cells were then examined for growth, and colonies arising were isolated and grown up for characterization.

Isolation and purification of plasmid and mammalian DNA

Plasmid and mammalian DNA was isolated and purified using methods outlined by Maniatis *et al.* (1982). The genomic DNA was sheared before precipitation by bringing the DNA up and down through a 20-gauge needle twice.

Co-transfection of CHOMG cells with mammalian genomic DNA and the pSV₂-neo plasmid

Transfection experiments were carried out using the calcium phosphate procedure (Gorman, 1985) with slight modifications. Recipient CHOMG cells were seeded at a density of 5×10^5 cells/100 mm dish 24 h before treatment. At 3 h before treatment, fresh media was added to all dishes. The DNA (40 μ g of sheared HT-29 DNA and 5 μ g of pSV₂-neo per dish) was co-precipitated in a solution of 0.25 M-CaCl₂ to which an equal volume of a double-strength Hepes-buffered saline solution (NaCl, 16.0 g/l; KCl, 0.74 g/l; Na₂HPO₄, 0.2 g/l; dextrose, 2.0 g/l; Hepes, 10.0 g/l; pH 7.1) was added dropwise while the solution was slowly aerated. Approx. 24 h after the addition of 1 ml of DNA co-precipitate to each dish, media was replaced and cells were allowed to grow for 48 h. Control dishes received the same treatment without HT-29 DNA.

The first selection was begun 48 h after the removal of the precipitate. The cells were exposed to the neomycin analogue G-418 (800 μ g/ml). The expression of the pSV₂-neo drug-resistance marker was used to identify those cells which were competent for the stable uptake and expression of exogenous DNA during the transfection procedure (Wigler *et al.*, 1977). Cell death was evident within 7–10 days, and colonies representing drug-resistant cells arose around day 14 in transfections which had included pSV₂-neo.

The second selection was initiated after the G-418-resistant colonies were very evident. The cells were pooled and replated at $(0.3\text{--}5) \times 10^5$ cells/100 mm dish in the presence of 5.0 mM-DFMO and 2.0 mM-aminoguanidine. After 3 days the media was changed to contain 5.0 mM-DFMO, 2.0 mM-aminoguanidine, 50 μ M-putrescine and 5 μ M-spermine. These cells were maintained under these selective conditions for up to 3 weeks. When colonies

arose and grew to sufficient size, they were picked and grown up for characterization.

Uptake measurements

The cells were plated in multiwell plates. After the indicated growth period, the growth media was aspirated off and α -MEM (without FBS) containing labelled uptake substrate was added. The cells were then incubated at 37 °C in 10% CO₂ and 93% humidity. After the appropriate incubation time, the media was aspirated off and the cell washed twice with 1 ml of ice-cold α -MEM (without FBS) containing 20 mM unlabelled substrate and then twice with 1 ml of ice-cold PBS. The washed cells were dissolved by sonication in 0.1 M-NaOH, and aliquots of this solution were used for protein quantification and for determination of radioactivity. Protein was measured by the dye-binding method of Bradford (1976), with the dye from Bio-Rad. Radioactivity was determined by using a Beckman LS-3133T scintillation counter after the addition of 10 ml of ACS II (Amersham-Searle). Uptake measurements were carried out under conditions in which uptake was known to be linear with respect to time. Where the effect of DFMO on polyamine-uptake activity was quantified, cells were exposed to a 5.0 mM dose of this drug for 48–72 h before uptake measurements.

RESULTS

When grown under the standard culture conditions, CHO and CHOMG cells were equally sensitive to exposure to DFMO, both with respect to depletion of intracellular polyamines (results not shown) and to the inhibition of cellular proliferation (Table 1). However, as shown in Table 1, although the antiproliferative effects of DFMO were completely reversed by provision of exogenous putrescine, spermidine or spermine in the CHO cells, these compounds were totally unable to promote increased growth in the CHOMG mutants. Even 500 μ M-putrescine did not significantly restore cell growth in DFMO-treated CHOMG cells. The more lipid-soluble acetylated analogue of spermidine, N¹-acetyl-spermidine, was able to increase growth of CHO cells to a limited extent, but not that of CHOMG cells. However, N⁸-acetylspermidine had no positive effect on either cell line (Table 1).

Populations of CHO and CHOMG cells were morphologically distinguishable within 24 h of plating in the presence of 5 mM-DFMO and 50 μ M-putrescine. Exposure to this selective medium resulted in CHOMG cells which were elongated and narrow relative to control CHOMG cells or CHO cells in either media. Such an appearance is typical in CHO and CHOMG cells treated with DFMO alone. Single colonies of CHO or CHOMG cells were easily identified in this way within 72 h of exposure to DFMO and putrescine. Cells could also be readily distinguished on the basis of growth in DFMO plus putrescine. When an equal number of wells in a 96-well plate were plated with either one CHO or one CHOMG cell in the presence of 5 mM-DFMO and 50 μ M-putrescine, colonies were evident in 36 wells after 6 days. All of these colonies arose from CHO cells. These experiments indicated that cells expressing putrescine transporters can readily be distinguished from those which do not transport putrescine by growth in a medium containing putrescine plus DFMO.

Table 1. Ability of exogenous polyamines to reverse DFMO-induced cytostasis in CHO and CHOMG cells

CHO and CHOMG cells were plated at a density of 4×10^4 per 35 mm dish and grown for 72 h in the presence of medium containing 5.0 mM-DFMO and 2.0 mM-aminoguanidine. This medium was replaced with medium containing these drugs and the polyamine concentration shown. After a further 72 h, the number of cells present in the dishes containing polyamines was determined and expressed as a percentage of the number of cells in dishes exposed only to DFMO and aminoguanidine as shown. These values are the means $\pm$ S.D. of cell numbers in three to five dishes. The actual cell numbers for the control DFMO-treated cultures without polyamine addition were 32×10^4 for CHO cells and 26×10^4 for CHOMG cells. When the cells were cultured in the absence of DFMO, the numbers after 3 days were 220×10^4 for CHO cells and 250×10^4 for CHOMG cells.

Addition	Cell number (as % of DFMO control)	
	CHO cells	CHOMG cells
5 μ M-Putrescine	1210 $\pm$ 53	82 $\pm$ 8
25 μ M-Putrescine	1190 $\pm$ 73	111 $\pm$ 12
50 μ M-Putrescine	1238 $\pm$ 70	90 $\pm$ 14
500 μ M-Putrescine	N.D.*	121 $\pm$ 17
5 μ M-Spermidine	1080 $\pm$ 40	92 $\pm$ 11
50 μ M-Spermidine	1021 $\pm$ 47	106 $\pm$ 13
5 μ M-Spermine	1055 $\pm$ 26	89 $\pm$ 11
50 μ M-Spermine	496 $\pm$ 18	105 $\pm$ 15
N ⁸ -Acetylspermidine	128 $\pm$ 26	100 $\pm$ 14
N ¹ -Acetylspermidine	250 $\pm$ 25	86 $\pm$ 24

* Abbreviation: N.D., not determined

In order to test whether this selection could be carried out in the presence of a large excess of the transport-deficient cells, a small number of CHO cells were mixed with CHOMG cells (1 or 10 in 3×10^4) and cells grown for 72 h in the presence of 5 mM-DFMO. At this time, the medium was changed to contain 5 mM-DFMO and 50 μ M-putrescine and the cell growth was monitored for a further 72 h. At this time, discrete colonies were found in the dishes grown from the mixture of CHO and CHOMG cells, but there were no colonies from dishes containing only CHOMG cells. When representative colonies were examined for polyamine uptake and for sensitivity to MGBG, their properties were confirmed to be those of CHO cells in that they readily took up putrescine or spermine and were highly sensitive to the toxic effects of MGBG (Table 2). It has been shown that the toxic drug MGBG is a substrate for polyamine transporters and that CHOMG cells, which do not transport polyamines, are more than a thousand times less sensitive to MGBG than are parental CHO cells (Mandel & Flintoff 1978; Byers *et al.*, 1987).

The successful selection of polyamine-transport-positive cells described above indicated that a similar scheme should prove useful in the isolation of a CHOMG transfectant containing a foreign polyamine-transport gene. Human colon-tumour HT-29 cells were used as a source of external DNA. These cells have an active uptake system for putrescine and for spermine, although

Table 2. MGBG-sensitivity and polyamine uptake in CHO, CHOMG and cells selected by growth in DFMO and putrescine (clones C1 and D1)

Representative clones, C1 and D1, which were selected for growth in DFMO and putrescine, were obtained from a mixture of 1 CHO cell in 10^4 CHOMG cells as described in the Materials and methods section, and their sensitivity to MGBG and ability to take up polyamines compared. For measurement of MGBG-sensitivity, the cells were plated at 6×10^5 cells/30 mm dish in the presence or absence of 10 μ M-MGBG. At 72 h, the cells were harvested and counted. The values reported are the means $\pm$ S.D. for at least three observations. For polyamine-uptake measurements, cells were grown in 24-well plates. While the cells were still in exponential phase, the growth medium was removed and replaced with uptake medium which contained 20 μ M-[¹⁴C]putrescine or 20 μ M-[¹⁴C]spermine and incubated for 45 min. These uptake values are the means $\pm$ S.D. for at least three observations.

Cells	MGBG-sensitivity ($10^{-6} \times$ cell no.)		Uptake of polyamines (nmol/h per mg of protein)	
	Control	+ 10 μ M-MGBG	Putrescine	Spermine
CHO	6.5 $\pm$ 0.3	NVC*	22.5 $\pm$ 0.6	4.9 $\pm$ 0.3
CHOMG	4.1 $\pm$ 0.2	4.0 $\pm$ 0.2	< 0.4	< 0.2
Clone C1	4.3 $\pm$ 0.2	NVC	22.4 $\pm$ 0.8	5.1 $\pm$ 0.2
Clone D1	4.9 $\pm$ 0.6	NVC	23.4 $\pm$ 0.6	5.8 $\pm$ 0.3

* Abbreviation: NVC, no viable cells.

the activity is lower than that present in CHO cells (see Table 3). CHOMG cells were co-transfected with genomic HT-29 DNA and pSV₂-neo plasmid DNA. These cells were selected with the neomycin analogue G-418 to obtain a sub-population of cells which were competent for the uptake and stable expression of foreign DNA at the time of transfection. These cells were then selected for the ability to grow in the presence of 5 mM-DFMO supplemented with 50 μ M-putrescine and 5 μ M-spermine. A colony was apparent 6 days after the addition of polyamines. Cells grown up from this isolated colony were examined and found to be MGBG-sensitive and to take up radiolabelled polyamines (results not shown). These cells were morphologically indistinguishable from CHO and CHOMG cells. These cells were cloned and this clone, designated 'CHOMGT-1', was used for a more detailed analysis of the polyamine-uptake system of these cells.

CHOMGT-1 cells clearly expressed characteristics consistent with the activity of a functional polyamine-transport system. These cells were MGBG-sensitive, although slightly less so than CHO cells (Fig. 1). Direct evidence for polyamine transport by these transfectants was provided by their ability to take up radiolabelled putrescine and spermine (Fig. 2). The $V_{\max}$ values for putrescine and spermine transport in the CHOMGT-1 cells were lower than those observed for uptake by the CHO cells, but the apparent K_m values were comparable (Table 3). Depletion of intracellular polyamines by inhibiting ODC activity has been reported to increase polyamine uptake in a variety of mammalian cell lines (Alhonen-Hongisto *et al.*, 1980; Seppänen *et al.*, 1981;

Table 3. Polyamine-uptake properties of CHOMG cell clones transfected with human DNA

Polyamine transport and $V_{\max}$ and apparent K_m values for all of the CHO and CHOMGT cells were determined as described in the legend to Fig. 2. Similar procedures were used for the HT-29 cells, but in these cells there was a significant non-saturable uptake of putrescine at higher putrescine concentrations.

Cell	Polyamine substrate	K_m (μM)	$V_{\max}$ (nmol/h per mg of protein)
CHO	Putrescine	4.5	15.6
	Spermine	0.7	4.8
CHOMG	Putrescine	—	—
	Spermine	—	—
HT-29	Putrescine	4.6	0.6
	Spermine	0.8	0.9
CHOMGT-1	Putrescine	4.4	6.3
	Spermine	0.6	2.6
CHOMGT-3	Putrescine	2.4	3.5
	Spermine	0.4	1.9
CHOMGT-6	Putrescine	3.1	5.7
	Spermine	0.6	2.9
CHOMGT-9	Putrescine	3.7	10.4
	Spermine	1.0	4.4
CHOMGT-10	Putrescine	14.0	2.8
	Spermine	0.5	0.4
CHOMGT-11	Putrescine	—	—
	Spermine	—	—
CHOMGT-12	Putrescine	7.9	14.2
	Spermine	2.2	5.5
CHOMGT-13	Putrescine	1.7	0.16
	Spermine	18.8	0.3

Rinehart & Chen, 1984; Kramer *et al.*, 1985; Byers *et al.*, 1987). DFMO pretreatment increased the $V_{\max}$ for putrescine and spermine uptake by both CHO and CHOMGT-1 cells with no change of the apparent K_m (Fig. 2). Polyamine transport in both cell types was reduced by exposure to exogenous polyamines (results not shown).

CHOMGT-1 cells were tested for proline dependency to confirm their CHOMG origin, as CHO and CHOMG cell represent the only proline auxotrophs presently used in this laboratory. CHOMGT-1 cells were found to be proline-dependent, with growth reduced by 90% in the absence of proline and restored to normal values when proline was provided. The CHOMGT-1 cells exhibited G-418 resistance by reaching confluence in 4 days, despite exposure to G-418 at 800 $\mu\text{g}/\text{ml}$. Non-transfected CHO and CHOMG cells under these conditions showed signs of toxicity at day 4 and were all dead by day 6.

Growth curves for CHO, CHOMG, and CHOMGT-1 cells are shown in Fig. 3. At early stages the growth rates of these cell lines were identical, but the CHO and CHOMG exponential growth slowed substantially as the cells reached confluence, whereas CHOMGT-1 cells continued to grow exponentially, despite high cell density.

Since only one transfectant cell line which clearly expressed a polyamine-transport system was obtained in the first transfection, the procedure was repeated. In this

Fig. 1. Sensitivity of CHO and CHOMGT-1 cells to MGBG

CHO (●) and CHOMGT-1 (○) cells were plated at 4×10^5 cells/100 mm dish in the presence of MGBG at the concentrations indicated and the cell number determined 72 h later. The number of cells in the dishes containing MGBG was compared with those in control dishes. Results are the means $\pm$ S.D. for three dishes.

case, 90 dishes corresponding to 4.5×10^7 cells were used for the transfection with HT-29 and pSV-neo DNA. In this case a total of 20 colonies were found. These colonies were cloned by growing from single cell density, and clonal lines were established from all of the cells which, by visual inspection, showed some sensitivity to the growth inhibition by exposure to 10 μM -MGBG. Cells from five of the colonies selected did not show any sensitivity, and were discarded, a further eight failed to grow under the cloning conditions, and seven clones were obtained (CHOMGT-3, 6, 9, 10, 11, 12 and 13). These cells were examined for their response to MGBG (Table 4), uptake of putrescine and spermine (Table 3) and growth properties (Fig. 4).

As shown in Table 4, all of these lines except CHOMGT-11 and CHOMGT-13 were much more sensitive to MGBG than were the CHOMG cells. Lines CHOMGT-6, -9 and -12 were similar to the CHO cells, lines CHOMGT-1 and -3 were possibly slightly less sensitive, but line CHOMGT-10 was clearly less sensitive, having an IC_{50} (concentration causing 50% inhibition) value 40 times greater than CHO cells (but still 30 times less than CHOMG cells).

All of the transfectant cell lines clearly accumulated polyamines, except for line CHOMGT-11 (Table 3). Determination of the apparent K_m and $V_{\max}$ for putrescine and spermine indicated that all of the transfectants differed in these properties from both CHO cells and from HT-29 cells. Transfectants CHOMGT-1, -3, -6, -9 and -12 were quite similar to each other in that the $V_{\max}$ values were in the range 3.5–14.2 nmol/h per mg for putrescine and 1.9–5.5 nmol/h per mg for spermine, whereas the apparent K_m values were 2.4–7.9 μM for putrescine and 0.4–2.2 μM for spermine (Table 2). CHOMGT-10 and CHOMG-13 differed more strikingly from the other transfectants. CHOMGT-10 had a high K_m and a low $V_{\max}$ for putrescine uptake and a low K_m and $V_{\max}$ for spermine. The uptake of putrescine and spermine by CHOMGT-13 was very low, but clearly detectable, and the K_m for spermine was very high.

The growth properties of the additional transfectant

Fig. 2. Effect of DFMO treatment on putrescine and spermine uptake by CHO and CHOMGT-1 cells

CHO cells (*a* and *b*) or CHOMGT-1 cells (*c* and *d*) were plated at 4×10^4 cells/well in control medium (○) or at 1×10^5 cells/well in medium containing 5 mM-DFMO (●) in 24-well plates. At 72 h after plating the growth medium was replaced with α -MEM containing [14 C]putrescine (*a* and *c*) or [14 C]spermine (*b* and *d*) and the uptake of these amines was determined. Results are shown as means $\pm$ S.D. for three observations. The apparent K_m was determined by using an Eadie-Hofstee plot of this data. Cultures of both cell lines were found to contain identical protein-per-cell values, indicating that the expression of the results as nmol/h per mg of protein does allow for valid comparisons of the cellular ability to accumulate polyamines.

Fig. 3. Growth curves for CHO (●), CHOMG (■) and CHOMGT-1 (□) cells

Cells were plated at 3×10^5 cells/dish in 60 mm dishes. At the times indicated, the dishes were washed twice with 5 ml of PBS at 37 °C and trypsin-treated for 5 min, after which media containing FBS was added. The cells were gently scraped from the dish with a rubber policeman, dispersed by pipetting and counted on a haemocytometer. Results are shown $\pm$ S.D. for three to five dishes. Where no error bar is shown, the variation is less than the size of the symbol.

Table 4. MGBG-sensitivity of CHOMG-cell clones transfected with human DNA

Cell	Sensitivity to MGBG (IC_{50} , μM)
CHO	0.12
CHOMG	160
HT-29	2.5
CHOMGT-1	0.24
CHOMGT-3	0.30
CHOMGT-6	0.16
CHOMGT-9	0.18
CHOMGT-10	5.0
CHOMGT-11	200
CHOMGT-12	0.12
CHOMGT-13	165

CHO-cell clones expressing the human polyamine-transport system are shown in Fig. 4. Clone CHOMGT-3 resembled CHOMGT-1 in growing to a much higher density than the CHO cells, but the other clones did not show this property, and clones CHOMGT-6, -11, -12 and -13 actually grew slightly more slowly than the control CHO or CHOMG cells.

The clones of CHOMG cells which expressed poly-

Fig. 4. Growth curves for CHOMG transfectants expressing human polyamine-transport protein

Cell growth curves were set up as in Fig. 3, except that the medium was changed at 72 h and at 144 h. Results are shown in (a) for CHOMGT-3 (○), CHOMGT-6 (●), CHOMGT-12 (□) and CHOMGT-9 (■) cells and in (b) for CHO (○), CHOMGT-11 (●), CHOMGT-13 (□) and CHOMGT-10 (■) cells. Results are shown as the means of counts from three separate dishes, which agreed within $\pm 5\%$.

amine transport after transfection with human DNA are unlikely to represent a reversion of the CHOMG mutation. A number of experiments were carried out in which no colonies of cells expressing polyamine transport were obtained from the CHOMG cells. In six separate experiments in which CHOMG cells were transfected with HeLa DNA, a total of 1.6×10^8 cells were plated, and no cells competent for polyamine transport were identified. Although the reasons for the failure of these experiments are unknown, the fact that no polyamine-transport-positive cells arose suggests that a change reversing the CHOMG mutation is an extremely infrequent event. Furthermore, no polyamine-transport-positive cells arose in the second selection in an experiment in which 6×10^5 CHOMG cells were transfected with pSV₂-neo without any human-cell DNA, selected for G-418 resistance, and then exposed to DFMO plus polyamines.

DISCUSSION

The isolation of seven distinct clones of CHOMG cells capable of polyamine transport from the transfection of CHOMG cells with human DNA clearly demonstrates that the procedure devised for the isolation and identification of cells possessing a functional polyamine-transport system is satisfactory for this purpose. The single clone

which was isolated by this screening procedure and which did not transport polyamines (CHOMGT-11) probably results from an overproduction of ODC. Such overproduction, which occurs quite readily in cells maintained in the presence of DFMO and is often the result of amplification of the ODC gene, allows cells to produce polyamines and grow even in the presence of the inhibitor of polyamine biosynthesis (reviewed by Pegg, 1986, 1988; McCann *et al.*, 1987). This possibility can readily be examined, and such clones eliminated, by testing that the growth in DFMO requires the addition of an exogenous polyamine (T. L. Byers & A. E. Pegg, unpublished work.).

Although it is not possible to totally eliminate the possibility that the CHOMG clones expressing polyamine transport are revertants of a mutated hamster-cell gene rather than expression of a human gene, this is extremely unlikely for a number of reasons. Firstly, no clones positive for polyamine transport were obtained in transfections with the pSV₂-neo plasmid alone, and no such revertants have been observed over a considerable period of time in which the CHOMG cells have been in experimental use. Also, it should be noted that the properties of the positive clones are not exactly the same as those of the wild-type CHO cells. Finally, a first selection with G-418 was used to identify the population of cells competent for uptake, incorporation and stable expression of DNA during the transfection, and thus reduce the number of cells which must be screened to identify the desired transfectant. This protocol also minimizes the possibility of selecting a revertant mutant, in that the frequency of transformation for two markers is higher than the frequency of transformation for one marker along with spontaneous mutation for the second (Rubin *et al.*, 1983). Definitive proof that a human transport protein is expressed in the hamster cells will have to await the cloning of this protein, but the present system provides a good model system in which this cloning can be carried out. The isolation of secondary transfectants and the production of cosmid libraries from such transfected cells are required.

It has been suggested that there may be a multiplicity of polyamine-transport carrier proteins with overlapping specificities (Byers *et al.*, 1987). This is consistent with the observations on polyamine transport made by Porter *et al.* (1984) and by Feige & Chambaz (1985). The present results are consistent with this hypothesis, since not all of the transfectant clones have the same properties, although these results could also be explained by truncation or reduced expression of the human protein in some of the clones. However, it is noteworthy that there is a rough correlation between MGBG sensitivity and the uptake of putrescine in that those cells with a high capacity for putrescine uptake are the most sensitive to MGBG (Tables 3 and 4). This finding is consistent with the hypothesis that MGBG uptake is predominantly via the carrier which is primarily concerned with putrescine uptake. Genetic analysis also indicates that more than one locus controls MGBG uptake in CHO cells (Heaton & Flintoff, 1988). The existence of a number of proteins needed for polyamine transport is suggested by these results, and it cannot be ruled out that the loss of polyamine transport on the CHOMG cells is due to a mutation in a critical regulatory protein rather than the carrier itself. However, the complete absence of polyamine transport in these mutants would support the

concept that a membrane transport protein is altered and that this is restored by the expression of the human gene.

The abnormal growth properties of the first transfectant expressing polyamine transport seem unlikely to be related to the expression of the human transport system, since they were not seen in clones CHOMGT-6, -9, -10, -12 and -13. Clones 6, 9 and 12 in particular are quite similar to clones 1 and 3 in the uptake of putrescine and spermine, but did not show the growth to higher saturation densities shown by clones 1 and 3. On the other hand, it is unlikely that the presence of two clones having these growth properties among the eight clones isolated occurred by chance. It is possible that the gene for the polyamine-transport protein is located sufficiently close to a gene allowing this growth pattern, so that there is a significant chance that they will be transferred together. Another factor which should be taken into consideration is that the selection procedure used may discriminate in favour of cells with this growth pattern, since the colonies picked are those growing among a background of cells arrested by exposure to DFMO. A third, perhaps less likely, possibility is that the gene for a normal polyamine-transport protein may have been truncated during the transfection process and thus be expressed as an aberrant protein in CHOMGT-1 and CHOMGT-3 cells. It is conceivable that the changes in polyamine uptake in cells reaching confluence are part of a complex signalling mechanism which halts proliferation; abnormal expression of a protein involved in this signal may result in transformation. There are independent reports which support a possible link between transformation and altered polyamine transport. Mutations reducing MGBG uptake in F₄ adenovirus-transformed rat cells have been shown to also significantly reduce properties associated with transformation (Sircar *et al.*, 1987; Rodrigues *et al.*, 1987). Wallace & Keir (1982) reported that baby-hamster kidney cells transformed by polyoma virus exhibit aberrant polyamine uptake and excretion characteristics; putrescine accumulation was enhanced and polyamine excretion was minimal in the transformed cells. Transformation of rat-1 cells following transfection with the N-myc gene increases the accumulation of the bis-ethyl analogues of spermidine and spermine (Chang *et al.*, 1988). The isolation of secondary transfectants should enable these possibilities to be tested.

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