Protein turnover, growth and proliferation in CHO cells

Variation within and between mutant classes for salvage pathway enzymes

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We have examined the clonal variation in rates of amino acid transport, protein synthesis, protein degradation, growth and proliferation for CHO cells with mutations in the purine and pyrimidine salvage pathways. First we compared three clonal cell lines, each with a different mutation, with the heterozygous parental line AT3-2. Overall, the correlation between rates of protein turnover, growth and proliferation was excellent. The slower growth and proliferation of one mutant, AB3 (TK⁻, APRT⁻), is explained by a low intrinsic rate of protein synthesis coupled with a smaller response in rates of amino acid transport, protein synthesis and protein degradation to insulin, serum and dexamethasone. Secondly, we compared seven aza-adenine-resistant and 14 thioguanine-resistant mutants of AT3-2 and found significant differences in control and insulin-stimulated rates of protein turnover both within and between mutant populations. A significant difference between the populations was unexpected because each individual cell line was cloned from a spontaneous pre-existing mutation in AT3-2, and each population should have the same average rate. Remarkably, all 24 mutants had lower rates of protein synthesis than AT3-2. We cannot explain the data solely in terms of mutations in the salvage pathways. Rather, we propose that the mutant survivors have randomly down-regulated the intrinsically fixed growth factor-regulated pathways of protein turnover, resulting in a broad spectrum of lower metabolic rates.

INTRODUCTION

The growth and proliferation of cultured cells requires the co-ordinated regulation of macromolecular synthesis and degradation. For example, amino acid or serum deficiency arrests cell growth, and these cells have characteristically low rates of amino acid transport, protein synthesis and DNA synthesis, and high rates of protein degradation (Hershko *et al.*, 1971; Sander & Pardee, 1972; Castor, 1977; Ballard & Gunn, 1982; Baserga, 1985). Conversely, serum or purified serum growth factors added to cultured cells rapidly increase rates of amino acid transport and protein synthesis, and decrease rates of protein degradation (Hershko *et al.*, 1971; Hollenberg & Cuatrecasas, 1975; Holley, 1980; Ballard & Gunn, 1982, 1985; Gunn *et al.*, 1983). As a result, rates of protein accumulation (synthesis minus degradation) and the total protein content increase as a prelude to DNA synthesis and cell division (Baxter & Stanners, 1978; Ballard, 1982; Ballard & Gunn, 1985; Baserga, 1985). While the mechanism for controlling and co-ordinating protein turnover and growth will be the same in different cell types, specific differences between cell types in rates of protein synthesis and degradation are found and should be anticipated (Baxter & Stanners, 1978; Cockle & Dean, 1982; Ballard & Gunn, 1982, 1985).

However, the question of what is the variation in rates of protein turnover for closely related (even isogenic) cell lines arising from a single cell population has not been addressed. Here we compare rates of protein synthesis, protein degradation, growth and proliferation in CHO mutants selected by clonal outgrowth of drug-resistant survivors in media containing 8-aza-adenine (AA), 6-thioguanine (TG) or 5-bromodeoxyuridine (BUdR). We examine the variation both within and between

these closely related populations to determine whether or not rates of protein turnover are intrinsically fixed and invariant.

MATERIALS AND METHODS

Cell culture

The four CHO cell lines, AT3-2, 2E5, T2S24 and AB3, were a gift from Dr. Rodney Nairn and Dr. Gerald Adair, University of Texas System Cancer Center, Smithville, TX, U.S.A. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % calf serum. The AT3-2 line (Adair *et al.*, 1980, 1983) is heterozygous for adenine phosphoribosyltransferase (APRT) and thymidine kinase (TK), allowing single-step selection of mutants resistant to AA (APRT locus), BUdR (TK locus) and TG [at the X-linked hypoxanthine phosphoribosyltransferase (HGPRT) locus]. Using these agents, the CHO cell lines 2E5 (homozygous deficient for TK) (Nairn *et al.*, 1982) and T2S24 (homozygous deficient for both APRT and HGPRT) (Nairn *et al.*, 1988) were derived from AT3-2 by clonal outgrowth in selection medium. The AB3 line (homozygous deficient for both APRT and TK) was selected in BUdR from an AA-resistant clone of AT3-2. Single mutants of AT3-2 resistant to either AA (designated AA^R sub-lines 1–7) or TG (designated TG^R sub-lines 1–14) were isolated as described (Carver *et al.*, 1980). Briefly, AT3-2 cells (5×10^5 cells per 100 mm Petri dish) were incubated in DMEM containing 10 % dialysed calf serum and either 0.66 mM-AA or 26 μ M-TG. Once visible colonies formed they were picked, dispersed with trypsin, transferred to individual T-25 flasks and grown to confluency in selection medium. Stock cultures were maintained in selection medium, but the selection pressure was removed for approx. three generations when cells were passaged to 24-well multiwell dishes

Abbreviations used: AA, 8-aza-adenine; AIB, 2-aminoisobutyric acid; APRT, adenine phosphoribosyltransferase (EC 2.4.2.7); BUdR, 5-bromodeoxyuridine; CHO, Chinese hamster ovary; DMEM, Dulbecco's modification of Eagle's minimal essential medium; DegMEM, DMEM containing 2 mM-leucine; HGPRT, hypoxanthine phosphoribosyltransferase (EC 2.4.2.8); K_{RNA} , protein synthesis per unit RNA (μ g/4 h per μ g of RNA); TG, 6-thioguanine; TK, thymidine kinase (EC 2.7.1.21).

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for experiments. Calf serum, DMEM and trypsin were purchased from Irvine Scientific, and tissue culture flasks and dishes were from Costar. Insulin was Novo Actrapid, L-[4,5-³H]leucine (40–60 Ci/mmol) and 2-amino[1-¹⁴C]isobutyric acid (40–60 mCi/mmol) were from the Amersham Corporation, and all other reagents were from Sigma.

Growth curves

Cells were plated at $(6-8) \times 10^4$ cells/well into 6-well multiwell dishes in DMEM containing 10% calf serum, and 6 h later the medium was changed to one containing 1% serum. After 18 h (Day 0) the medium was changed to DMEM containing either 1% or 10% calf serum. On Day 0 and days thereafter the cell monolayer was dispersed with EDTA/trypsin and the number of cells determined with a Coulter Counter.

Measurement of protein turnover

Rates of intracellular protein turnover were measured by a pulse-chase technique as described previously (Ballard, 1982; Ballard & Gunn, 1985). Briefly, growth of cells in 24-well multiwell dishes was arrested by incubation in leucine-free DMEM containing 1% calf serum and [³H]leucine (1 μ Ci/ml) as the only sources of leucine. After 18 h, the monolayers were rinsed twice with Earle's Salts containing 2 mM-leucine and incubated in DMEM containing 2 mM-leucine (DegMEM). After a further 2 h, the medium was changed to DegMEM containing additions as described and the cells were incubated for a 4 h measurement period. Rates of protein degradation (% in 4 h) are reported as the percentage of radioactivity soluble in 10% trichloroacetic acid. Rates of protein synthesis were measured using exactly the same protocol, except that [³H]leucine (2 μ Ci/ml) is added to the DegMEM during the 4 h measurement period. Leucine incorporation from the medium (specific radioactivity 1 μ Ci/ μ mol) was calculated as nmol/4 h per mg of protein. Dexamethasone (0.1 μ M) was added at the beginning of the 18 h label/sham-label period, and was present throughout the experiment; serum (1%) and insulin (0.1 μ M) were added during the 4 h measurement period.

From the asymptote of [³H]leucine incorporation into cell protein measured over a 48 h labelling period, we calculated the maximum incorporation of leucine from the medium to be 577 ± 14 nmol/mg of protein. This value is less than the leucine content in cell protein (Ballard & Gunn, 1985), presumably because (1) unlabelled leucine is present in cell protein at the start of the labelling period, (2) some of the leucine derived by intracellular protein degradation is reincorporated, and (3) unlabelled leucine is derived by proteolysis of serum proteins. To correct for these factors, and at the same time express protein synthesis in the same units as protein degradation (i.e. % synthesized in 4 h), here we report fractional rates of protein synthesis, calculated as:

$$\frac{100 \times \text{nmol/4 h per mg of protein}}{577 \text{ nmol/mg of protein}}$$

The fractional rates of protein accumulation or protein growth (% in 4 h) are calculated as the difference between the amount synthesized (%) and the amount degraded (%). Because synthesis and degradation are measured in separate multiwell dishes, there are two or three fewer independent calculations for accumulation than there are measurements of synthesis and degradation. It should be noted that degradation is measured relative to the labelled proteins present at the start, and synthesis is relative to the labelled proteins present at the end of the 4 h measurement period. Hence synthesis is underestimated relative to degradation. However, the error is small unless the monolayers are accumulating significant amounts of protein during the 4 h period

or, in comparisons between cell lines, one line is accumulating significantly more protein than the other. In addition, the degradation protocol specifically removes the short-lived rapidly degraded proteins (comprising 4–6% of the proteins labelled in the 18 h pulse) and measures the loss of the long-lived slowly degraded proteins, and therefore will slightly underestimate rates of degradation. Similarly, the synthesis protocol will also slightly underestimate rates, because proteins with short half-lives turn over during the 4 h period. Thus the procedures measure relative rates of turnover. Nevertheless, the sum of the errors involved must be small because, as shown below in Table 1, there is an excellent correlation between calculated and observed rates of protein growth.

As an independent estimate of protein synthesis we measured the RNA (Munro & Fleck, 1969) and protein (Smith *et al.*, 1985) content of the cells, and calculated the RNA/protein ratio (μ g of RNA/mg of protein). Then the synthesis rate per unit of RNA (μ g of protein/4 h per μ g of RNA) is calculated as:

$$K_{\text{RNA}} = \frac{1000 \times \text{fractional rate of protein synthesis}}{100 \times \mu\text{g of RNA/mg of protein}}$$

For these experiments the protocol and treatments were exactly as described for the turnover experiments, except that the cells were grown in 6-well multiwell dishes.

Measurement of 2-aminoisobutyric acid (AIB) uptake

Rates of AIB transport were measured as previously described (Helm & Gunn, 1987). Briefly, cells in 24-well multiwell dishes were incubated overnight in DMEM plus 1% calf serum, rinsed twice with Earle's Salts and incubated in DMEM with or without insulin (0.1 μ M). After 2 h, the medium was changed to an identical one and the incubation continued for another 3.5 h, after which the medium was changed to Earle's Salts with or without insulin as before. After a further 30 min incubation the monolayer was pulse-labelled by addition of 1 μ Ci of [¹⁴C]AIB (0.6 mM final) for 10 min. The monolayer was then rinsed with cold phosphate-buffered saline (20 mM-sodium phosphate/0.9% NaCl, pH 7.3) containing 10 mM-AIB and dissolved in 0.1 M-NaOH/0.1% Triton X-100. Duplicate samples were taken for determination of radioactivity and protein.

Statistical analysis

Analysis of variance (ANOVA) procedures at the 95% confidence level were performed using either the Kramer modification of Duncan's New Multiple Range Test to compare differences between cell lines, or Dunnett's post-hoc procedure for comparing treatments with a control. The program was SuperANOVA v1.11 (Abacus Concepts, Inc.) run on a Macintosh SE/30. Linear regression analysis at the 95% confidence level was used to determine the difference in proliferation rates of the four cell lines.

RESULTS

Comparisons between four CHO cell populations

Proliferation and protein turnover. We have measured differences in proliferation and protein turnover between three closely related CHO cell lines, namely 2E5 (TK^{-/-}), T2S24 (APRT^{-/-}, HGPRT^{-/-}) and AB3 (APRT^{-/-}, TK^{-/-}), selected from a common parental population of AT3-2 cells heterozygous for APRT and TK. One mutant, AB3, had much lower rates of proliferation (Fig. 1). In medium containing 10% serum, T2S24, AT3-2 and 2E5 grew at the same rate, with doubling times of 0.57, 0.60 and 0.65 day respectively; AB3 grew significantly more slowly, with a doubling time of 1.01 days (see legend to Table 1).

Fig. 1. Growth curves for the four CHO mutants

The increases in cell numbers for AT3-2 ($\square$), 2E5 ($\triangle$), T2S24 ($\circ$) and AB3 ($\bullet$) cell lines were measured daily for 3 days for growth in 10% serum or every other day for 8 days for growth in 1% serum, as described in the Materials and methods section. Each point is the mean of three observations.

Table 1. Calculated and observed doubling times for CHO cells

The rate constants (k) and doubling times (T_D) were calculated either from the rates of protein accumulation in 1% serum (Table 2) or from the change in cell number during growth in 1% serum (Fig. 1). Data are the means $\pm$ S.E.M. The rate constants for growth in 10% serum (Fig. 1) were (day^{-1}): AT3-2, 1.15 ± 0.03^a ; 2E5, 1.07 ± 0.04^a ; T2S24, 1.22 ± 0.04^a ; AB3, 0.69 ± 0.05^b . Values with different superscript letters are significantly different ($P < 0.01$).

Cell type	Calculated		Observed	
	k (day^{-1})	T_D (days)	k (day^{-1})	T_D (days)
AT3-2	0.32 ± 0.05^{ab}	2.14	0.37 ± 0.03^{ab}	1.87
2E5	0.31 ± 0.04^{ab}	2.20	0.33 ± 0.03^{bc}	2.12
T2S24	0.36 ± 0.03^a	1.94	0.43 ± 0.02^a	1.61
AB3	0.21 ± 0.01^b	3.25	0.23 ± 0.02^c	2.96

In medium containing 1% serum, doubling times were approx. 3-fold longer at 1.61, 1.87, 2.12 and 2.96 days for T2S24, AT3-2, 2E5 and AB3, respectively, with AB3 growing significantly more slowly than T2S24 and AT3-2 (Table 1). To investigate the metabolic basis for this difference, we have determined the effect of anabolic and catabolic conditions on growth by measuring rates of total protein synthesis and degradation.

Rates of protein turnover as a function of cell density and serum concentration for the faster growing T2S24 and the slower growing AB3 are shown in Fig. 2. Qualitatively the density-dependent changes observed were similar for both cell lines. In serum-containing media, rates of protein synthesis declined in a density-dependent fashion, whereas rates of protein degradation were independent of cell density. Thus, as the cells approach confluency, rates of protein accumulation and growth (Fig. 1) are limited by a decrease in protein synthesis, not by an increase in protein breakdown. Under step-down conditions in serum-free medium (DegMEM in Fig. 2) there was a decrease in rates of protein synthesis and a dramatic increase in rates of protein

Fig. 2. Rates of protein synthesis and degradation in T2S24 and AB3 cell lines as a function of cell density

T2S24 and AB3 cells were plated at 1×10^4 cells/well into 24-well multiwell dishes and grown in DMEM containing 10% calf serum. Rates of protein synthesis ($\circ$) and degradation ($\square$) ($\%$ in 4 h) were determined daily as described in the Materials and methods section with DegMEM alone, or together with either 1% or 10% calf serum added as indicated during the 4 h measurement period. Results are means $\pm$ S.E.M. of three observations.

degradation. This response was especially evident in sparse cultures, was manifest at all cell densities and was density-dependent in magnitude. Quantitatively the major difference between the two cell lines was the lower rate of protein synthesis measured in sub-confluent cultures of AB3. This was found in serum-free medium as well as in media containing either 1% or 10% serum. Rates of protein degradation at any density and rates of protein synthesis at confluency were generally the same for both cell lines. Therefore, compared with T2S24, growth and proliferation in AB3 will be limited by its lower rate of protein synthesis.

Regulation of protein turnover. Further experiments comparing rates of protein turnover in the four CHO mutants were performed in sub-confluent cultures. The range of cell densities (μg of protein/ cm^2) in these experiments were: AT3-2, 31–64; 2E5, 41–63; T2S24, 28–54; AB3, 29–55. The data (Table 2) are a compilation of nine experiments separately measuring the effects of insulin, serum and dexamethasone relative to a control in serum-free medium, and are presented together to emphasize the differences between cell lines. The insulin and serum groups were chosen as two anabolic conditions relative to the serum-free control and dexamethasone groups, which represented two catabolic conditions.

All four CHO lines, in keeping with a variety of other mammalian cells in culture, exhibited qualitatively the same responses to anabolic and catabolic conditions. Serum (1%)

Table 2. Effects of serum, dexamethasone and insulin on rates of protein turnover in the four CHO mutants

Rates of protein synthesis, degradation and accumulation (% in 4 h) were measured as described in the Materials and methods section. Data are presented as the means $\pm$ S.E.M. for 21 (control, insulin), 24 (dexamethasone) and 9 (serum) observations for synthesis and degradation, and 7, 8 and 3 observations respectively for accumulation. For comparisons between treatment groups within a cell line, means marked with an asterisk are significantly different from the control with no addition ($P < 0.05$). For comparisons between cell lines within a treatment group, means with the same superscript letter are not significantly different ($P > 0.05$).

Addition	Cell type	Synthesis (%/4 h)	Degradation (%/4 h)	Accumulation (%/4 h)
Serum	AT3-2	12.09 $\pm$ 0.35 ^a	6.55 $\pm$ 0.22 ^{*a}	5.54 $\pm$ 0.91 ^{ab}
	2E5	12.02 $\pm$ 0.33 ^{*a}	6.64 $\pm$ 0.17 ^{*a}	5.39 $\pm$ 0.17 ^{*ab}
	T2S24	12.16 $\pm$ 0.18 ^{*a}	6.03 $\pm$ 0.31 ^{*a}	6.13 $\pm$ 0.51 ^{*a}
	AB3	9.92 $\pm$ 0.15 ^{*b}	6.31 $\pm$ 0.09 ^{*a}	3.62 $\pm$ 0.24 ^{*b}
Insulin	AT3-2	12.81 $\pm$ 0.12 ^{*a}	5.56 $\pm$ 0.15 ^{*a}	7.25 $\pm$ 0.45 ^{*a}
	2E5	12.40 $\pm$ 0.17 ^{*ab}	5.69 $\pm$ 0.10 ^{*ab}	6.72 $\pm$ 0.22 ^{*a}
	T2S24	12.02 $\pm$ 0.20 ^{*b}	5.69 $\pm$ 0.18 ^{*ab}	6.33 $\pm$ 0.45 ^{*a}
	AB3	9.91 $\pm$ 0.07 ^{*c}	6.03 $\pm$ 0.07 ^{*b}	3.87 $\pm$ 0.13 ^{*b}
None	AT3-2	11.75 $\pm$ 0.16 ^a	7.31 $\pm$ 0.13 ^a	4.44 $\pm$ 0.45 ^a
	2E5	10.89 $\pm$ 0.16 ^b	8.09 $\pm$ 0.12 ^b	2.80 $\pm$ 0.34 ^{bc}
	T2S24	10.85 $\pm$ 0.13 ^b	7.50 $\pm$ 0.12 ^a	3.35 $\pm$ 0.26 ^b
	AB3	9.45 $\pm$ 0.10 ^c	7.47 $\pm$ 0.05 ^a	1.99 $\pm$ 0.16 ^c
Dexamethasone	AT3-2	10.89 $\pm$ 0.08 ^{*a}	7.86 $\pm$ 0.12 ^{*a}	3.03 $\pm$ 0.21 ^{*a}
	2E5	9.48 $\pm$ 0.07 ^{*b}	8.51 $\pm$ 0.08 ^{*b}	0.97 $\pm$ 0.08 ^{*b}
	T2S24	9.76 $\pm$ 0.11 ^{*c}	7.99 $\pm$ 0.08 ^{*a}	1.77 $\pm$ 0.19 ^{*c}
	AB3	8.96 $\pm$ 0.09 ^{*d}	7.80 $\pm$ 0.06 ^{*a}	1.16 $\pm$ 0.12 ^{*b}

Table 3. Effects of serum, dexamethasone and insulin on the translational phase of protein synthesis in the four CHO mutants

The total RNA and protein contents were determined as described in the Materials and methods section. The K_{RNA} values, or the protein synthesis rates per unit of RNA, were calculated using the fractional rates of protein synthesis reported in Table 2. Data are the means $\pm$ S.E.M. of six determinations. For comparisons between treatment groups within a cell line, values marked with an asterisk are significantly different from the control with no addition ($P < 0.05$). For comparisons between cell lines within a treatment group, means with the same superscript letter are not significantly different ($P > 0.05$).

Addition	Cell type	Protein content (μg of protein/ cm^2)	RNA content (μg of RNA /mg of protein)	K_{RNA} (μg of protein/4 h per μg of RNA)
Serum	AT3-2	27.57 $\pm$ 0.94	19.17 $\pm$ 0.38 [*]	6.32 $\pm$ 0.12 ^a
	2E5	30.80 $\pm$ 1.67	18.40 $\pm$ 0.54	6.56 $\pm$ 0.19 ^a
	T2S24	27.13 $\pm$ 1.93	18.79 $\pm$ 0.49	6.49 $\pm$ 0.17 ^a
	AB3	43.01 $\pm$ 0.98	17.74 $\pm$ 0.49	5.61 $\pm$ 0.17 ^b
Insulin	AT3-2	27.31 $\pm$ 0.68	19.67 $\pm$ 0.32 ^{*a}	6.52 $\pm$ 0.11 ^a
	2E5	30.52 $\pm$ 1.59	18.86 $\pm$ 0.63 ^{ab}	6.61 $\pm$ 0.22 ^a
	T2S24	28.41 $\pm$ 2.21	18.86 $\pm$ 0.32 ^{ab}	6.38 $\pm$ 0.11 ^a
	AB3	43.25 $\pm$ 0.72	18.00 $\pm$ 0.16 ^b	5.51 $\pm$ 0.05 ^b
None	AT3-2	25.30 $\pm$ 0.94	17.88 $\pm$ 0.36	6.59 $\pm$ 0.13 ^a
	2E5	30.02 $\pm$ 1.62	17.93 $\pm$ 0.47	6.10 $\pm$ 0.16 ^b
	T2S24	26.07 $\pm$ 2.30	17.72 $\pm$ 0.29	6.13 $\pm$ 0.10 ^b
	AB3	38.85 $\pm$ 0.71	17.48 $\pm$ 0.35	5.42 $\pm$ 0.12 ^c
Dexamethasone	AT3-2	24.52 $\pm$ 1.17	18.77 $\pm$ 0.21	5.81 $\pm$ 0.06 ^{*a}
	2E5	27.82 $\pm$ 1.96	18.28 $\pm$ 0.86	5.24 $\pm$ 0.23 ^{*b}
	T2S24	24.61 $\pm$ 2.49	18.66 $\pm$ 0.48	5.25 $\pm$ 0.14 ^{*b}
	AB3	35.87 $\pm$ 1.02	17.61 $\pm$ 0.27	5.10 $\pm$ 0.08 ^b

significantly altered rates of protein turnover in each cell line: it inhibited rates of protein degradation and stimulated rates of protein synthesis, with the net result that rates of protein accumulation increased (Table 2). The four CHO mutants all responded to insulin in a concentration-dependent fashion, with half-maximum effects on protein turnover occurring at 1 nM in each case (results not shown). Rates of protein accumulation for insulin-treated cells were some 2-fold higher than for controls (Table 2) because of significant and opposing effects of the hormone to increase rates of protein synthesis and decrease rates

of protein degradation in each cell line, with the greater effect being on breakdown rather than synthesis. Dexamethasone had a net catabolic effect on protein accumulation by decreasing rates of protein synthesis and increasing rates of protein degradation in all four CHO lines (Table 2). The maximum catabolic effect of dexamethasone on protein turnover was achieved at 6–24 h after addition (results not shown). Note how for insulin (compared with the control) and for dexamethasone (compared with either the control or serum), modest (range 5–34%) but opposite changes in rates of protein synthesis and breakdown are trans-

Table 4. Effect of insulin on rates of AIB transport in the four CHO mutants

Data are presented as the means $\pm$ S.E.M. for a total of 10 independent observations in two experiments. Δ Insulin is the difference between insulin-treated and control groups for the two experiments. Data in parentheses are the percentage increases in AIB transport produced by insulin. For the comparison between cell lines, means with the same superscript letter are not significantly different ($P > 0.05$).

Cell line	AIB transport (nmol/10 min per mg of protein)		
	Control	Insulin	Δ Insulin
AT3-2	8.80 $\pm$ 0.11 ^a	10.28 $\pm$ 0.20 ^{ab}	1.48 $\pm$ 0.50 ^{ab} (16.9 $\pm$ 5.8)
2E5	9.14 $\pm$ 0.40 ^a	10.55 $\pm$ 0.28 ^b	1.41 $\pm$ 0.33 ^{ab} (16.2 $\pm$ 5.7)
T2S24	8.95 $\pm$ 0.19 ^a	11.40 $\pm$ 0.15 ^c	2.45 $\pm$ 0.19 ^a (27.5 $\pm$ 3.2)
AB3	8.68 $\pm$ 0.27 ^a	9.64 $\pm$ 0.28 ^a	0.96 $\pm$ 0.06 ^b (11.1 $\pm$ 0.3)

lated into large and significant anabolic or catabolic effects on rates of accumulation (Table 2).

Quantitatively, there are major differences between the cell lines. In particular, AB3 has an intrinsically lower rate of protein synthesis under catabolic (serum-free medium and dexamethasone treatment) and anabolic (serum and insulin treatment) conditions. At the same time, its rate of protein breakdown generally is no different from that of the other three cell lines. Thus significantly lower rates of protein accumulation in AB3 arise because rates of protein synthesis are consistently low, and not because rates of protein breakdown are consistently high. Significant variation in rates between the other three lines was also seen. For example, rates of protein synthesis in 2E5 and T2S24 were intermediate between those in AT3-2 and AB3 for control, insulin and dexamethasone treatments, while rates of protein degradation in 2E5 for control and dexamethasone treatment were higher than in the other cell lines. As a result, rates of protein accumulation under the two catabolic conditions were lower for 2E5, T2S24 and AB3 than for AT3-2. However, AB3 is unique in having the lowest rate of protein synthesis for all treatments. The net result of this intrinsically low rate is that protein accumulation in AB3 was significantly decreased under all four conditions (Table 2) as well as across the growth curve

(Fig. 2). Indeed, we calculated the doubling times for total cell protein in medium containing 1% serum to be 1.94, 2.14, 2.20 and 3.25 days for T2S24, AT3-2, 2E5 and AB3 respectively, values that correlate precisely ($r^2 = 0.95$) with the independent measure for rates of proliferation in 1% serum (Table 1).

Capacity and efficiency of protein synthesis. As an independent estimate of protein synthesis, we calculated the (total RNA)/(total protein) ratio, an indicator of the capacity for protein synthesis, and then determined K_{RNA} , the protein synthesis rate in terms of RNA, an indicator of the efficiency of protein synthesis (see Sugden & Fuller, 1991, for a recent review). The general trend was for serum and insulin to significantly increase the RNA/protein ratio compared with the control ($P < 0.05$). Average values (irrespective of cell type) were ($\mu\text{g}/\text{mg}$): control, 17.75 $\pm$ 0.18; serum, 18.53 $\pm$ 0.25; insulin, 18.85 $\pm$ 0.22; dexamethasone, 18.33 $\pm$ 0.26. However, individually this was only significant for AT3-2 treated with serum and insulin (Table 3). Compared with the control, the K_{RNA} was significantly decreased by dexamethasone ($P < 0.05$). Average values were (μg of protein/4 h per μg of RNA): control, 6.06 $\pm$ 0.11; serum, 6.25 $\pm$ 0.11; insulin, 6.25 $\pm$ 0.11; dexamethasone, 5.35 $\pm$ 0.09. Individually, an effect of dexamethasone was seen with all cell lines except AB3 (Table 3).

In comparing the cell lines, the overall RNA/protein ratio was significantly lower in AB3 ($P < 0.05$). This is because, compared with the other cell lines, the ratio for AB3 is significantly lower under anabolic conditions (1% serum or insulin; $P < 0.05$) but not under catabolic conditions. Average values were ($\mu\text{g}/\text{mg}$): AT3-2, 18.87 $\pm$ 0.21; 2E5, 18.37 $\pm$ 0.31; T2S24, 18.51 $\pm$ 0.21; AB3, 17.71 $\pm$ 0.16. The K_{RNA} was also significantly lower in AB3 compared with the other three cell lines in the control, serum and insulin groups, and compared with AT3-2 in the dexamethasone group (Table 3). Thus AB3 has a lower capacity, a lower efficiency and a lower rate of protein synthesis.

Amino acid transport. We measured rates of protein synthesis by pulse-labelling in 2 mM-leucine. We assumed that this floods the intracellular pool so that leucine transport and the intracellular leucine concentration do not limit rates of protein synthesis. Leucine is transported by System L (Collarini & Oxender, 1987), and transport of other amino acids by other transport systems may be limiting protein synthesis in AB3. Accordingly, we have measured the effect of insulin on rates of

Table 5. Incremental effects of dexamethasone, insulin and serum on rates of protein turnover in the four CHO mutants

Data were calculated from Table 1 as 'treatment - control' ($\Delta\%$ in 4 h) so that anabolic effects are positive for synthesis (Δ Synthesis), negative for degradation (Δ Degradation) and positive for the difference between synthesis and degradation (Δ Effect). Values are the means $\pm$ S.E.M. of eight (dexamethasone), seven (insulin) and three (serum) observations. For comparisons between cell lines within a treatment group, means with the same superscript letter are not significantly different ($P > 0.05$).

Addition	Cell type	Change (%/4 h)		
		Δ Synthesis	Δ Degradation	Δ Effect
Dexamethasone	AT3-2	-0.86 $\pm$ 0.23 ^{ab}	0.55 $\pm$ 0.19 ^a	-1.41 $\pm$ 0.22 ^{ab}
	2E5	-1.41 $\pm$ 0.15 ^b	0.42 $\pm$ 0.15 ^a	-1.83 $\pm$ 0.18 ^a
	T2S24	-1.09 $\pm$ 0.46 ^{ab}	0.49 $\pm$ 0.07 ^a	-1.58 $\pm$ 0.40 ^a
	AB3	-0.49 $\pm$ 0.08 ^a	0.33 $\pm$ 0.17 ^a	-0.82 $\pm$ 0.22 ^b
Insulin	AT3-2	1.06 $\pm$ 0.13 ^a	-1.75 $\pm$ 0.17 ^a	2.80 $\pm$ 0.22 ^a
	2E5	1.51 $\pm$ 0.24 ^a	-2.40 $\pm$ 0.21 ^b	3.91 $\pm$ 0.40 ^b
	T2S24	1.17 $\pm$ 0.24 ^a	-1.81 $\pm$ 0.17 ^a	2.98 $\pm$ 0.33 ^a
	AB3	0.46 $\pm$ 0.12 ^b	-1.45 $\pm$ 0.15 ^a	1.90 $\pm$ 0.16 ^c
Serum	AT3-2	0.34 $\pm$ 0.06 ^a	-0.76 $\pm$ 0.12 ^a	1.10 $\pm$ 0.11 ^a
	2E5	1.13 $\pm$ 0.15 ^b	-1.45 $\pm$ 0.12 ^b	2.58 $\pm$ 0.16 ^b
	T2S24	1.31 $\pm$ 0.13 ^b	-1.47 $\pm$ 0.09 ^b	2.78 $\pm$ 0.18 ^b
	AB3	0.47 $\pm$ 0.13 ^a	-1.16 $\pm$ 0.20 ^{ab}	1.64 $\pm$ 0.33 ^a

Fig. 3. Rates of protein synthesis and degradation within the (a) AA^R and (b) TG^R mutant populations

The selection of the 7 AA^R and 14 TG^R mutants and procedures for measuring rates of protein synthesis and degradation are described in the Materials and methods section. A total of six separate protein turnover experiments was performed and each of the 21 mutants was included in two or three of the experiments. AT3-2 was carried through each experiment (data reported in Table 6). The cell densities for the AA^R mutants were 17–50 μg of protein/ cm^2 and for the TG^R mutants they were 25–50 μg protein/ cm^2 . The data are plotted as synthesis (abscissa) versus degradation (ordinate) for control (○) and insulin-treated (□) cells. Differences in rates of protein synthesis or degradation within a population are given by the separation along the abscissa or ordinate respectively. Results are means $\pm$ S.E.M. of 4–6 independent observations on a single mutant sub-line.

AIB transport as an indicator of System A transport activity (Table 4). Control rates of AIB transport were the same in each cell line, at ≤ 1 nmol/min per mg of protein. Insulin significantly increased AIB transport in all four mutants, but the insulin-stimulated rate in AB3 was lower, and in T2S24 was higher, than those in AT3-2 and 2E5. Both the absolute and relative increases were significantly greater for T2S24, the mutant with the shortest doubling time, than for AB3, the mutant with the longest doubling time. Overall, the hierarchy is T2S24 > 2E5 $\geq$ AT3-2 $\geq$ AB3. Thus the correlation between proliferation, growth and protein synthesis extends to amino acid transport.

Response to anabolic and catabolic regulators. The small effect of insulin on AIB transport in the AB3 cell line points out another major difference between the cell lines, namely that AB3 displays a decreased response to anabolic and catabolic conditions. Absolute differences in rates between treatment and control are reported in Table 5; relative changes are not reported but may be calculated from the data in Tables 2 and 5. Both the relative and absolute changes in rates of protein synthesis,

breakdown and accumulation are often decreased in AB3 compared with one or more of the other three cell lines. For example, the percentage changes in rates of protein synthesis with the insulin, serum and dexamethasone treatments compared with the control were significantly decreased in AB3 compared with 2E5 and T2S24, and the percentage change in the rate of protein degradation for insulin treatment was also significantly decreased in AB3 compared with 2E5 ($P < 0.05$). Similarly, the absolute effect of dexamethasone in AB3 was significantly lower than in 2E5 and T2S24, due entirely to a smaller effect on rates of protein synthesis. Insulin had a significantly smaller effect in AB3 compared with the other three cell lines because of a decreased response of both the synthesis and breakdown pathways, whereas the effect of serum was significantly lower in AB3 compared with 2E5 and T2S24 because of a smaller effect on rates of protein synthesis. It is interesting to note that both the relative and incremental effects of serum in AT3-2 were the same as in AB3, yet AB3 had a longer doubling time, presumably due to its lower intrinsic rate of protein synthesis (Table 2). Thus AB3 had not only a lower rate of protein synthesis but also a decreased response to hormones. Overall, we conclude that pleiotropic responses exhibit significant clonal variation between sub-populations of cells.

Comparisons within CHO cell populations

It simply might be fortuitous that each of these four lines were separately selected and picked from a drug-resistant population of similar mutants. Thus the clonal variation we have measured between mutant classes may be simply a reflection of the variation within a mutant class. To test this hypothesis we selected seven clones of AT3-2 resistant to AA (AA^R sub-lines 1–7) and 14 clones resistant to TG (TG^R sub-lines 1–14), and measured the influence of insulin on rates of protein turnover in each. Even though these are not independent populations of cells, we still found significant variation both within and between the mutant classes. To clearly illustrate the differences within mutant classes we present the data as a scatter plot (Fig. 3). Rates of protein synthesis within the AA^R sub-clones differed significantly, ranging from 8.12 to 10.15 % per 4 h and from 8.88 to 10.92 % per 4 h in control and insulin-treated cells respectively. Similarly, rates of protein breakdown varied from 7.05 to 8.17 % per 4 h in control and from 5.34 to 5.88 % per 4 h in insulin-treated cells. For the TG^R sub-lines the variation was even greater (Fig. 3). In control and insulin-treated groups respectively, rates of protein synthesis varied from 8.62 to 11.22 % per 4 h and from 10.03 to 12.30 % per 4 h, whereas rates of protein degradation varied from 6.05 to 8.11 % per 4 h and from 4.72 to 5.74 % per 4 h respectively. It is notable that sub-lines with the same rates of protein degradation may have significantly different rates of protein synthesis, and vice versa. Thus within a mutant population there may be significant variation in rates of protein turnover.

To examine the variation between mutant classes, we calculated the average rates of protein synthesis and degradation for the AA^R and TG^R mutants (each sub-line is represented once) and compared these population averages to the rates for AT3-2 measured in the same experiments (Table 6). We found that average rates of protein degradation in the AA^R group were significantly higher than in the AT3-2 or TG^R groups under control conditions, and significantly higher than in the TG^R group after insulin treatment. Rates of protein synthesis in the AA^R and TG^R groups were significantly lower than in the AT3-2 line, and also significantly different from each other for both control and insulin-treated cells. Thus rates of protein accumulation in AA^R and TG^R cells were significantly different from each other and significantly lower than in the parental population.

Table 6. Population differences in rates of protein turnover for the AA^R and TG^R mutants of AT3-2

The average rates of protein synthesis, degradation and accumulation (% in 4 h) for the individual AA^R and TG^R sub-lines shown in Fig. 3 were combined to obtain the population averages and compared with rates determined for AT3-2 in the same experiments. The average cell densities were 34, 43, and 44 µg of protein/cm² for AA^R, TG^R and AT3-2 respectively. Data are the means ± S.E.M. of 6, 7 and 14 determinations for AT3-2, AA^R and TG^R respectively. Values in parentheses are the percentage changes relative to the control (no addition). For comparisons between cell lines within treatment groups, means with the same superscript letter are not significantly different ($P > 0.05$).

Addition	Mutant	Change (%/4 h)		
		Synthesis	Degradation	Accumulation
None	AT3-2	12.21 ± 0.05 ^a	6.85 ± 0.08 ^a	5.36 ± 0.12 ^a
	AA ^R	9.46 ± 0.29 ^b	7.46 ± 0.14 ^b	1.95 ± 0.37 ^b
	TG ^R	10.12 ± 0.17 ^c	6.91 ± 0.16 ^a	3.21 ± 0.29 ^c
Insulin	AT3-2	13.26 ± 0.27 ^a (8.6 ± 2.0)	5.27 ± 0.20 ^{ab} (23.1 ± 2.4)	7.99 ± 0.41 ^a
	AA ^R	10.36 ± 0.28 ^b (9.4 ± 0.9)	5.64 ± 0.07 ^a (24.3 ± 1.0)	4.72 ± 0.30 ^b
	TG ^R	11.27 ± 0.18 ^c (11.6 ± 0.7)	5.19 ± 0.10 ^b (24.5 ± 0.8)	6.08 ± 0.24 ^c

The incremental effect of insulin on protein turnover also showed considerable variation within a mutant class. Again, to illustrate this point clearly, we present the data as a scatter plot (Fig. 4), in which the effect of insulin on either protein synthesis or degradation is plotted as a function of the total effect. The incremental insulin effect on breakdown exceeded that on synthesis indicating that, using the DegMEM control as a baseline, insulin had a greater effect on rates of protein degradation than on protein synthesis in these CHO mutants (see also Table 5). Indeed, within each mutant class there were no significant differences in the absolute effects of insulin on protein synthesis, whereas there were significant differences for the effect on protein breakdown ($P < 0.05$). Insulin stimulated protein synthesis and inhibited protein breakdown by 7–13 % and 21–31 % respectively within the AA^R sub-lines, and by 7–16 % and 19–30 % respectively within the TG^R sub-lines (results not shown). Again, significant differences within each population were found for these relative effects of insulin on protein breakdown. Consequently the total response (Δ Effect in Fig. 4) to insulin varied significantly from 2.3 to 3.7 % per 4 h within the AA^R sub-lines ($P < 0.05$) and from 2.0 to 3.9 % per 4 h within the TG^R sub-lines ($P < 0.05$). These differences can be explained by the normal variation within a population, as there were no significant differences in either the relative or the incremental effects of insulin on synthesis, degradation and accumulation between the AA^R, TG^R and AT3-2 populations (see Table 6 and the legend to Fig. 4).

DISCUSSION

We have examined the variation in the co-ordinate regulation of protein turnover both within and between related CHO cell lines selected from a common heterozygous parental population (AT3-2). Drug-resistant variants of AT3-2 arise spontaneously and randomly as mutational events prior to drug selection (Carver *et al.*, 1980; Adair *et al.*, 1983). Pre-existing mutations in HGPRT, APRT and TK are cloned by selecting in TG, AA and BUdR respectively for survivors which cannot metabolize the drug. Such HGPRT⁻, APRT⁻ and TK⁻ sub-lines provide one way of examining the clonal variation in pleiotropic responses within the original population. We have eliminated heterogeneity within the cell populations by clonal selection of cells. Similarly, we have minimized both density-dependent effects, by comparing sub-confluent cultures, and cell cycle effects, by incubating cells in leucine-free low-serum medium.

We have assumed, *a priori*, that these mutations will have no

effect on total protein turnover and no effect on the relationship between growth and proliferation. In some respects this is true, because all of the mutants exhibited the same regulatory patterns for protein turnover. We found an excellent correlation between the predicted doubling time, based on rates of protein accumulation, and the measured doubling time, based on cell number (Table 1). In addition, rates of protein synthesis and degradation in all mutants responded to the well-documented anabolic effects of serum, insulin and other serum growth factors (Baxter & Stanners, 1978; Lockwood & Minassian, 1982; Ballard, 1982; Ballard & Gunn, 1982, 1985; Gunn *et al.*, 1983; Cockle & Dean, 1984), and to the catabolic effects of a serum-free medium and dexamethasone (Hopgood *et al.*, 1981; Ballard & Francis, 1983). Serum and insulin increased fractional rates of protein synthesis by increasing the RNA/protein ratio, while dexamethasone decreased rates by altering the K_{RNA} (Table 3). Similar effects have been reported *in vivo* and *in vitro* (see Sugden & Fuller, 1991). Both slow-growing and fast-growing mutants also displayed density-dependent changes in growth and protein turnover that are controlled by alterations in rates of protein synthesis rather than protein degradation. An excess of synthesis over degradation, even in confluent cultures, and rates of protein degradation that are independent of density are both characteristics of protein turnover in transformed rather than contact-inhibited cells (Lockwood & Minassian, 1981; Cockle & Dean, 1982, 1984; Ballard & Gunn, 1985). Regulation through alterations in rates of protein degradation occurs as a stop-growth response to nutritional and hormonal deprivation (Hershko *et al.*, 1971; Ballard & Gunn, 1982) and is thought to involve autophagosome formation (Ballard & Gunn, 1982). The ability of confluent cultures of different CHO mutants to dampen the increase in rates of protein turnover associated with serum-free conditions (Fig. 2) points to a co-ordinated regulatory mechanism which is retained by the mutants and in which the lower demand for synthesis in confluent cultures evokes a smaller demand for breakdown.

However, we also found that rates of protein turnover and the response to growth factors display significant variation both within and between different sub-populations derived from a single population. Some of this variation may be explained by the normal variation in the parental AT3-2 population. For example, both the relative and absolute effects of insulin on protein turnover in the AA^R and TG^R sub-lines (Fig. 4), although displaying significant differences within each mutant population, showed no differences between populations. We conclude that the differences in relative and incremental effects for serum,

Fig. 4. Incremental effects of insulin on protein turnover within the (a) AA^R and (b) TG^R mutant populations

Data were re-calculated from Fig. 3 as 'treatment minus control' ($\Delta\%/4\text{ h}$), as described in the legend to Table 5, and (ignoring sign) plotted as $\Delta\text{Synthesis}$ ($\circ$) or $\Delta\text{Degradation}$ ($\square$) versus ΔEffect . Values are the means of two or three observations. Error bars have been omitted for clarity. The S.E.M. values averaged 20% of the mean for $\Delta\text{Synthesis}$, 10% for $\Delta\text{Degradation}$ and 13% for ΔEffect (see Table 5). The population averages for $\Delta\text{Synthesis}$, $\Delta\text{Degradation}$ and ΔEffect respectively, were ($\%/4\text{ h}$): AA^R mutants, 0.90 ± 0.07 , -1.82 ± 0.10 and 2.77 ± 0.19 ($n = 7$); TG^R mutants, 1.16 ± 0.07 , -1.72 ± 0.09 and 2.87 ± 0.14 ($n = 14$); and AT3-2, 1.05 ± 0.25 , -1.58 ± 0.15 and 2.63 ± 0.34 ($n = 6$).

insulin and dexamethasone displayed by 2E5, T2S24 and AB3 (Tables 4 and 5) also reflect the normal variation in the AT3-2 parental population. Similarly, it may be argued that the different rates of protein synthesis and degradation within the AA^R and TG^R sub-lines (Fig. 3) also reflect the population variance. However, although we have tested only a small number (21) of sub-lines of two different mutants, it is noteworthy that there was significant variation between the two mutant populations, and between the mutant populations and AT3-2 (Table 6). Indeed, rates of protein synthesis in the AA^R and TG^R sub-lines were all below the limits measured for the control and insulin groups in AT3-2, whereas rates of protein breakdown overlapped the limits for AT3-2 (cf. Tables 2 and 6 with Fig. 3). Furthermore, neither 2E5 nor T2S24 nor AB3 displayed rates of protein synthesis higher than those of AT3-2 (Tables 2 and 3), although rates of protein breakdown again may be higher or lower than in the parental cell line. The fact that we did not select any cell from the AT3-2 population with a rate of protein synthesis higher than that of the AT3-2 population average was unexpected, and means that the differences cannot all be ascribed to population

variance but must be explained by clonal variation. We conclude that AA, TG and BUdR have selected not only for mutants in the salvage pathways but also for cells that exhibit low intrinsic rates of protein synthesis and clonal variation in rates of protein turnover. Thus rates of protein synthesis, protein breakdown, growth and proliferation are not intrinsically fixed and invariant for closely related cell populations, but rather display significant differences.

Of the cell lines examined, these effects were most noticeable in the AB3 line. Overall, AB3 had a lower fractional rate of protein synthesis than other cell lines examined (Table 2 and Fig. 2) as a result of a decrease in the efficiency (K_{RNA}) and capacity (RNA/protein ratio) of translation (Table 3). In addition, and in keeping with some of the other sub-lines examined, AB3 also had a lowered response to catabolic and anabolic hormones (Table 5). This decrease occurred in both the synthetic and degradative pathways, but was not confined simply to protein turnover, as the incremental effect of insulin on AIB transport was also decreased in AB3 (Table 4). In addition, lower rates of proliferation (Fig. 1, Table 1) point to a concomitant lowering of serum responses in multiple pathways. We conclude therefore that AB3, relative to isogenic sub-lines, is a clonal variant with a lower intrinsic rate of protein synthesis and a decreased response to external signals. The pleiotropic result is a significant decrease in rates of growth and proliferation.

The AB3 line ($APRT^{-/-}$, $TK^{-/-}$) is deficient in both the purine and pyrimidine salvage pathways. We find it intriguing that mutations either in the pyrimidine pathway (the $TK^{-/-}$ 2E5 line) or in the purine pathway (the $APRT^{-/-}$ HGPRT $^{-}$ T2S24 line, as well as the $APRT^{-/-}$ AA^R sub-lines and the HGPRT $^{-}$ TG^R sub-lines) often gave rates of protein synthesis intermediate between those of AB3 and the parental AT3-2 (Tables 2 and 3, Fig. 3). Indeed, with the exception of the serum group data, the correlation is good. However, the significant differences between the two purine pathway mutant populations of the AA^R and TG^R clones (Table 6) and the universal occurrence of low rates of protein synthesis both mean that we cannot explain the effects in AB3, nor the differences noted between the other lines, nor even the differences noted within the AA^R and TG^R sub-lines solely in terms of the salvage pathways. Rather, we must conclude that these purine and pyrimidine analogues are giving rise either to epigenetic effects as a result of secondary metabolism of the analogues or to more global and unpredictable somatic mutations. The net result is that a series of intrinsically fixed, growth factor-regulated, metabolic rates are reset randomly downwards. Clearly our assumption that rates of protein turnover will not be altered in salvage pathway mutants is wrong. It will be interesting to see if clonal variation is displayed by other cell lines, and if it occurs in the absence of xenobiotic compounds.

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