Effects of nutrient deprivation and differentiation on the expression of growth-arrest genes (*gas* and *gadd*) in F9 embryonal carcinoma cells

John V. FLEMING, Susan M. HAY, D. Nicholas HARRIES and William D. REES¹

The Rowett Research Institute, Greenburn Road, Bucksburn, Aberdeen AB21 9SB, Scotland

The growth-arrest genes (*gas* and *gadd*) are widely expressed during mammalian embryogenesis and may be useful as markers of nutritional stress in the embryo. F9 embryonal carcinoma cells have been used to characterize the effect of serum or amino acid deficiency on growth-arrest gene expression in a differentiating embryonic cell. The differentiation markers, homeobox B2 (HoxB2), collagen type IV and laminin B2, were not induced by growth arrest. Treatment with all-*trans* retinoic acid (RA) produced a dose-dependent increase in alkaline phosphatase activity, which was unchanged in lysine-deficient medium and reduced in low-serum medium. Low-serum medium also reduced HoxB2 expression. There was a transient 2–6-fold increase in mRNAs for C/EBP- β , *gadd153*/CHOP-10 and *gas5* genes 24 h after transfer to amino-acid-deficient media. The mRNAs for the

gas2 and *gas6* genes began to rise slowly by 5–10-fold after a delay of approx. 24 h. The transient increases did not occur in low-serum medium where there was a much smaller and slower increase. Differentiation caused 1–2-fold increases in *gas2*, *gas3* and *gas6* mRNA levels. The transient overexpression of *gas5*, *gadd153*/CHOP-10 and CCAAT-enhancer-binding protein- β , and the later expression of *gas6* mRNAs in response to amino acid deficiency, were not affected by differentiation. RA treatment increased the expression of *gas3* and caused *gas2* to be transiently overexpressed in amino-acid-deficient medium. Differentiation in serum-deficient medium did not significantly alter the levels of the growth-arrest gene mRNAs. These results show that in F9 cells the growth-arrest genes are expressed sequentially as a result of nutrient stress.

INTRODUCTION

There is increasing evidence that inadequate fetal development *in utero* can increase the susceptibility of the adult to a variety of diseases [1]. In the offspring of mothers fed a protein-deficient diet, some organs such as the brain are protected and develop normally at the expense of other tissues such as the liver [2]. Genes overexpressed in response to nutritional stress may be useful in identifying sensitive stem cells and the mechanisms underlying asymmetric fetal growth. Two groups of growth-sensitive genes, the growth-arrest-specific (*gas*) genes, isolated from growth-arrested 3T3 cells [3], and the growth-arrest and DNA-damage (*gadd*) genes isolated from Chinese-hamster ovary (CHO) cells [4], are possible candidates for this role. These genes are expressed at different times during both pre-implantation [5] and post-implantation development and appear to play a role in the growth of many organs, including the cardiovascular system, lungs and kidney [6–8]. The mRNAs for both *gas* and *gadd* gene families are increased in growth-arrested cells. Since growth arrest can be caused by the lack of a suitable mitogenic stimulus, or by nutrient limitation, these studies were undertaken to investigate the changes in growth-sensitive gene expression induced by different challenges in an *in vitro* model of the early embryo.

F9 embryonal carcinoma cells differentiate *in vivo* into a variety of cell types that resemble those found in the developing fetus [9], and have been widely used to investigate the mechanisms of differentiation [10]. Treatment of undifferentiated F9 cells with retinoic acid (RA) initiates the differentiation programme, committing the cells to differentiate into a primitive endoderm-

like phenotype [11,12]. A number of markers are available to determine the extent of differentiation. These include an increase in alkaline phosphatase (ALP) activity and the expression of the homeobox B2 (HoxB2), collagen type IV and laminin genes [13].

The *gas* and *gadd* genes code for an unrelated group of structurally diverse proteins with a variety of functions. The *gas2* and *gas3* gene products are found in the cytoskeleton and the plasma membrane respectively. Cells transfected with expression vectors carrying the *gas2* and *gas3* genes can undergo apoptosis under some conditions, and it has been suggested that both genes are components of pathways leading to cell death [14,15]. The product of the *gas6* gene is the ligand for the Axl receptor tyrosine kinase and may be associated with cell–cell interactions [16]. It has also been reported to act as an anti-apoptotic paracrine agent in 3T3 fibroblasts [17]. Growth-sensitive genes also control differentiation and the *gadd153*/CHOP-10 gene plays an important role in mediating interactions between nutrition and differentiation in tissues expressing members of the CCAAT-enhancer-binding protein (C/EBP) family of transcriptional activators [18–20]. The product of the *gadd153* gene (isolated from CHO cells [4] and also known as CHOP-10, isolated from mouse cells [21]) retains the leucine zipper domain found in other members of the C/EBP family, but has lost the DNA binding domain, changing its DNA binding specificity. By forming inactive heterodimers with other leucine zipper transcriptional activators CHOP-10 can act as a dominant negative regulator of gene expression and block C/EBP-induced differentiation [21].

This paper describes the effects of growth-arrest on the differentiation of F9 EC (embryonal carcinoma) cells and changes

Abbreviations used: RA, all-*trans* retinoic acid; CHO, Chinese-hamster ovary; ALP, alkaline phosphatase; C/EBP, CCAAT-enhancer-binding protein; FCS, fetal-calf serum; NCS, newborn-calf serum; g3pdh, glyceraldehyde 3-phosphate dehydrogenase.

¹ To whom correspondence should be addressed.

in the expression of members of the *gas*, *gadd153*/CHOP-10 and C/EBP- β genes. Two types of growth arrest show that mitogens and nutrients have contrasting effects on mRNA levels. Our results show that the growth-arrest genes can be divided into two categories, depending on the timing of their expression following amino acid deprivation.

MATERIALS AND METHODS

Cell culture

Mouse F9 embryonal carcinoma cells (American Type Culture Collection, CRL-1720) were cultured in Dulbecco's modified Eagle's essential medium, containing 10% (v/v) fetal-calf serum (FCS; Gibco). Lysine-deficient medium has been described previously [22]. F9 cell monolayers were cultured on gelatin-coated plates [23]. Cells (1.4×10^6) were seeded in 90 mm gelatin-coated dishes and, after overnight culture in complete medium, were transferred to test medium 48 h before harvest. F9 aggregates were prepared by plating cells (10^5 cells/ml) in untreated bacteriological-grade Petri dishes [9]. After overnight culture to allow the aggregates to form, differentiation was initiated by the addition of 10^{-6} M RA (Sigma).

Growth curves

Cells (5×10^3) were plated into each well of a 96-well plate and cultured in 50 μ l of medium, which was changed every 2 days. At appropriate times, plates were harvested by adding 1% (v/v) glutaraldehyde in PBS, incubating at room temperature for 20 min and then washing twice with 0.2 ml of PBS [24]. The cells were stained with 0.1% (v/v) Crystal Violet, washed extensively, and the A_{620} of each well was read using a multiwell plate reader.

ALP assay

F9 cells were seeded into 24-well tissue-culture dishes precoated with gelatin at 0.95×10^4 cells per well (control) or 3.8×10^4 cells per well (growth-arrested). The cell numbers were chosen so that at the end of the culture period the cells were partially confluent, and the density was similar in dividing and growth-arrested cells. After overnight culture in complete medium, the cells were transferred to test medium and differentiation was induced by adding RA (Sigma) dissolved in ethanol. Equivalent quantities of ethanol were added to the control cultures. To prepare the enzyme extract, each well was washed twice with 1 ml of PBS and the cells solubilized in 0.4 ml of a buffer containing 10 mM Tris/HCl (pH 7.5), 0.5 mM $MgCl_2$ and 0.1% (v/v) Triton X-100. The DNA content was estimated by mixing 0.1 ml aliquots of the extract or standards with 2.5 ml of a bisbenzimidazole solution (0.2 μ g/ml in PBS) and measuring the fluorescence at 455 nm following excitation at 350 nm [25]. ALP activity was measured by the method described previously by Gianni et al. [26]. Briefly, 0.02–0.05 ml of extract was added to 0.18 ml of reaction mixture that contained 0.22 mM $MgCl_2$, 20 mM *p*-nitrophenyl phosphate and 0.166 M Sigma 221 alkaline buffer solution (Sigma). The reaction was carried out at 20 °C and the reaction was followed by measuring the absorbance at 414 nm every 30–60 s. The reaction rate was calculated from the linear portion of the progress curve.

RNA isolation

Total RNA was isolated from cultures of F9 cells by a modification of the guanidine isothiocyanate/phenol method [27]. Cell monolayers were washed twice with PBS and dissolved in

2 ml of Tri-reagent (Sigma). Aggregates were harvested by centrifugation at 700 *g* for 2 min, washed with PBS and dissolved in 2 ml of Tri-reagent. RNA was prepared according to the manufacturer's instructions.

cDNA probes for the growth-arrest genes

Probe templates were prepared from the plasmids: pA5A4 (*gadd153* [4]), MSV/EBP- β (C/EBP- β [18]), 15c7/HoxB2 (HoxB2 [28]), pCIV-1-PE16 (Collagen type IV [29]) and pLambB2 (laminin B2 [30]). Probes for the *gas2*, *gas3*, *gas5* and *gas6* genes were prepared by PCR amplification (35 cycles) of cDNA prepared from growth-arrested 3T3 cells. The primers and conditions used have been described previously [5]. The PCR products were cloned into the vector pCR script using the Srf cloning kit (Stratagene) and their identities were confirmed by sequencing.

Northern analysis

Total RNA (20 μ g) was separated on a 1.2% (w/v) agarose gel. The gel was stained with ethidium bromide to confirm that equal amounts had been loaded before transfer to a nylon membrane (Boehringer). Probe templates were labelled with [α - 32 P]dCTP using a Megaprime labelling kit (Amersham). Hybridizations were carried out by standard protocols [31], and the blots were washed to high stringency in $0.1 \times$ SSC (0.15 M NaCl/0.015 M sodium citrate) + 1% (w/v) SDS at 65 °C. Blots were imaged on a wire proportional counter (Packard Instant Imager). The mRNAs were quantified by measuring the amount of radioactivity hybridizing to each band on the Northern blot. The specific activities of the probes were calculated from the hybridization to standard samples of denatured plasmid DNA applied to the nylon membrane, and hybridized with the Northern blots. Blots were corrected for loading by reprobing for glyceraldehyde 3-phosphate dehydrogenase (g3pdh) mRNA and 18 S ribosomal RNA.

RESULTS

F9 stem cell growth in different media

F9 teratocarcinoma stem cells cultured in complete medium, containing 10% FCS, divide with a doubling time of approx. 15 h. Reducing the FCS content (1% FCS) increased the doubling time to approx. 50 h. A medium containing 2% (v/v) newborn-calf serum (NCS) did not support cell division and there was no net growth (Figure 1a). Cell growth was also dependent on the availability of the essential amino acid L-lysine and the rate fell when the concentration of free lysine in the medium fell below 100 nmol/ml (Figure 1b). The lysine-deficient medium contained 19 nmol of free lysine per ml compared with 200 nmol of free lysine per ml in the complete medium. Culture in the deficient medium increased the doubling time to approx. 70 h. Transferring F9 cells to low-serum or lysine-deficient medium for 48 h failed to produce any detectable effect on cell viability, and cells transferred back to complete medium grew normally. Prolonged exposure of F9 cells to amino-acid-deficient or low-serum medium was detrimental. Approx. 65% of the DNA present on day 2 was lost by day 4, as the cells became detached from the dish.

RA-induced differentiation of F9 stem cells and the effect of growth arrest

RA treatment produces a 3- to 5-fold increase in the ALP activity of F9 cells, and this is a characteristic marker of the primitive

Figure 1 Effect of culture medium on the growth of F9 EC stem cells

(a) Effect of serum concentration. F9 cells were grown in media containing 10% FCS (◆), 1% FCS (▲) or 2% NCS (■). Cells were harvested at the indicated times and stained with Crystal Violet. (b) Effect of L-lysine concentration. Cells were grown in lysine-free medium containing 10% FCS, supplemented with additional L-lysine. After 48 h the cells were harvested and stained with Crystal Violet.

endodermal phenotype [12,26]. Figure 2 shows the changes in ALP activity in response to RA under different growth conditions. The serum- and amino-acid-deficient media produced a small but significant induction of ALP. The magnitude of this growth-arrest-dependent increase was similar before and after RA stimulation and was additive to the effect of RA. The dose-response in lysine-deficient medium was similar to the exponentially growing cells except that the RA-dependent activity was superimposed upon an elevated basal activity (Figure 2, triangular symbols). Low-serum medium containing 1% FCS did not change the dose-response curve, and it ran parallel to that of the control cells over the whole range tested (results not shown). Medium containing 2% NCS produced a significant decrease in the dose-response of RA that was most pronounced at high RA concentrations (Figure 2, square symbols, $p = 0.006$ at 10^{-6} M RA). This medium did not produce any increase in basal ALP activity.

The differentiation of F9 cells also induces the expression of the HoxB2, collagen type IV and laminin B2 genes. The effects of growth arrest on the mRNAs for these genes was investigated by Northern blotting (Figure 3). To quantify gene expression the blots were reprobbed for g3pdh and the mRNA levels expressed as a fraction of the g3pdh message (Table 1). Although the g3pdh protein is required by dividing and stationary cells, growth arrest caused small increases in its mRNA relative to the 18S ribosomal subunit. These were 38% in low-serum medium and 23% in lysine-deficient medium respectively. The expression of the HoxB2 gene is one of the earliest events in the differentiation of

Figure 2 Dose-response curve of RA-dependent ALP activity in F9 EC cells

Cells were cultured for 24 h in complete medium containing 10% FCS (◆), lysine-deficient medium (▲) or medium containing 2% NCS (■). The cells were then treated with the indicated concentrations of retinoic acid, and after a further 48 h of culture in the same media, the ALP activity was assayed: ordinate units, m-units/μg of DNA. Data are the average of four independent determinations $\pm$ S.D.

F9 cells [32], and the mRNA was induced within 24 h of exposing the cells to RA (Figure 3a, top panel). Growth arrest alone did not bring about expression of HoxB2. Low-serum medium caused a highly significant 65% drop in the HoxB2 mRNA level in RA-treated cells ($p < 0.005$). There was a small but insignificant increase in the mRNA in cells cultured in the lysine-deficient medium. The expression of C/EBP- β is also an early event in the differentiation of many cells. However, in the F9 stem cells this mRNA could be detected before RA treatment (Figure 3a, middle panel). The expression of the C/EBP- β gene was unchanged by RA treatment but was sensitive to growth arrest both before and after differentiation. Low-serum medium decreased expression by approx. 50%, whereas lysine deficiency increased the mRNA level by 1.5–3-fold. The increase in lysine-deficient cells was transient and the C/EBP- β mRNA levels had fallen back to those of the controls within 72 h.

The mRNAs for collagen type IV and laminin are markers of the terminal differentiation of F9 cells, and the expression of both genes was dependent on RA treatment (results not shown). The expression of collagen type IV and laminin B2 genes in normal and growth-arrested cells 5 days after RA treatment is shown in Figure 3(b). Culture in low-serum medium produced trivial changes in the steady state levels of the mRNAs for both collagen type IV and laminin B2. Growth arrest due to lysine deficiency produced a 2.5-fold increase in collagen type IV and a 1.5-fold increase in laminin B2 mRNA levels compared with the cells in complete medium.

Figure 3 Expression of markers of differentiation in F9 EC cells

(a) Early markers. F9 aggregates were cultured in: lanes a, control medium; lanes b, low-serum medium; and lanes c, lysine-deficient medium, for 24 h. On the left-hand side of the blot are F9 stem cells (—RA) and on the right-hand side (+RA) are cells that had been stimulated with 10^{-6} M RA 24 h earlier. The upper panel shows the hybridization of a HoxB2 probe, the middle panel the hybridization of a C/EBP-β probe, and the lower panel the same blot probed for g3pdh. (b) Late markers. RNA was harvested from cells grown for 5 days in: lane a, complete medium; lane b, low-serum medium; and lane c, lysine-deficient medium. The medium was changed daily to prevent loss of the cells. The upper panel shows the hybridization of a collagen type IV probe, the middle panel the hybridization of a laminin B2 probe, and the bottom panel the same blot probed for g3pdh.

Growth-arrest gene expression in F9 stem cells

Figure 4 shows the hybridization of probes for the growth-arrest genes to mRNA from monolayers of F9 stem cells. A quantitative analysis of expression relating the levels to those of g3pdh mRNA is shown in Table 2. F9 stem cells express *gas2*, *gas5*, *gas6* and CHOP-10 genes and the transcripts are of similar size to those found in mouse 3T3 cells. Growth arrest had no effect on the sizes of the transcripts with the exception of *gas5*, where in some experiments a smaller transcript of approx. 500 bases was seen, in addition to the 800-base transcript shown in Figure 4. The *gas3* mRNA levels were too low to be detected on the

Table 1 Expression of differentiation markers in growth-arrested cells

Quantitative analysis of the expression of differentiation markers in growth-arrested F9 cells. The mRNA levels were determined from Northern blots and expressed as pg of mRNA per pg of g3pdh message in the same sample. Data are the average of two experiments except for HoxB2, which is the average of five experiments. HoxB2 and C/EBP-β expression was measured 24 h after treatment with RA. Collagen type IV and laminin B2 expression was estimated 5 days after RA treatment.

		mRNA levels (pg of mRNA/pg of g3pdh)			n
		10% FCS	2% NCS	Lysine-deficient medium	
HoxB2	+ RA	0.129	0.031	0.158	5
C/EBP-β	— RA	0.162	0.111	0.298	2
	+ RA	0.178	0.094	0.263	2
Collagen type IV	+ RA	1.044	0.886	2.466	2
Laminin B2	+ RA	0.590	0.613	1.494	2

Figure 4 Representative Northern blots showing the expression of the *gas2*, *gas5*, *gas6* and CHOP-10 genes in F9 stem cells

RNA was recovered from cells cultured for 48 h in control (10%), low-serum (2% NCS) and lysine-deficient (—lys) media.

Northern blots but a signal was produced by reverse transcriptase PCR, suggesting that the gene was expressed at low levels (results not shown). The up-regulation of the growth-arrest genes was not specific to lysine deficiency, and similar patterns were observed when cells were cultured in media deficient in leucine or threonine. The steady state levels of the growth-arrest gene mRNAs covered a considerable range, with *gas5* expression being the highest and *gas6* the lowest (Table 2).

Table 2 Growth-arrest gene expression in F9 cells

A quantitative analysis of growth-arrest gene expression in F9 monolayers 48 h after transfer to the test media. The mRNA levels were determined from Northern blots and expressed as pg of mRNA per pg of g3pdh message in the same sample. Data are the average of four estimations $\pm$ S.D.

	mRNA levels (pg of mRNA/pg of g3pdh)		
	10% FCS	2% NCS	Lysine-deficient medium
<i>gas2</i>	0.086 $\pm$ 0.038	0.123 $\pm$ 0.026	0.273 $\pm$ 0.025
<i>gas5</i>	24.6 $\pm$ 1.5	17.3 $\pm$ 1.2	65.0 $\pm$ 2.4
<i>gas6</i>	0.013 $\pm$ 0.003	0.022 $\pm$ 0.003	0.018 $\pm$ 0.002
CHOP-10	0.227 $\pm$ 0.340	0.316 $\pm$ 0.079	0.333 $\pm$ 0.090

Figure 5 Time course of growth-arrest gene expression in aggregates of F9 stem cells

Cells were grown in complete medium (◆), medium containing 2% NCS (■), and lysine-deficient medium (▲). The levels of each mRNA were calculated from Northern blots, and corrected for loading by reprobing the blot for 18 S RNA. Results are expressed as the relative level, giving the exponentially growing cells an arbitrary value of unity. Results shown for *gas5* and *gas2* are the average of four separate experiments and the results for *gas6* and CHOP-10 are the average of two experiments.

Figure 5 shows the time courses of growth-arrest gene expression in F9 cells following transfer to low-serum (broken lines) or amino-acid-deficient media (dotted lines). The mRNA levels increased slowly as the culture progressed in control medium (Figure 5, solid lines), either as the medium became exhausted or because the cells were approaching confluence. The latter may be the more important factor as the basal mRNA levels were increased in cells plated at a higher density. The growth-arrest gene mRNAs were much more sensitive to lysine deficiency (Figure 5, dotted lines) than to serum deprivation (Figure 5, broken lines), and the low-serum medium only produced modest increases after prolonged periods of culture. The amino-acid-deficient medium rapidly increased the mRNAs for *gas5* and CHOP-10, which reached a maximum after 24 h

Figure 6 Growth-arrest gene expression during differentiation

F9 cell aggregates were cultured for 1, 2 and 3 days after treatment with 10^{-6} M RA in control medium (◆), medium containing 2% NCS (■), and lysine-deficient medium (▲). The levels of each mRNA were calculated from Northern blots and corrected for loading by reprobing the blot for 18 S RNA. The results are expressed as the relative mRNA level, giving the expression in the exponentially growing cells an arbitrary value of unity, except for *gas3* where the cells in control medium 24 h after the addition of RA are given a value of unity. The results shown are the average of three separate experiments except for *gas6* which is the average of two experiments.

and then declined, returning to normal after 72 h. The mRNAs for *gas2* and *gas6* responded more slowly to the lysine-deficient medium and did not increase until 48 h after transfer. The increase in all of the mRNAs ranged between 2- and 10-fold in individual experiments.

Growth-arrest gene expression during differentiation

The growth-arrest genes continue to be expressed during the early stages of differentiation and the relative levels of the mRNAs are shown in Figure 6. In complete medium the steady

state level of *gas2* was increased by 2–3-fold within 24 h of RA stimulation. The increase in *gas3* was larger and Northern blots revealed the presence of two transcripts (2.1 and 3.7 kb) similar to those seen in 3T3 cells. After RA treatment the mRNAs for *gas2* and *gas3* remained elevated at a relatively constant level. There was also an increase in the *gas6* mRNA but this was slower and more progressive, rising by approx. 50 % in the 3 days after RA treatment. There was no change in *gas5* mRNA during differentiation. In cells growing normally, stimulation with RA caused small transient increases in the mRNAs for both C/EBP- β and CHOP-10 genes in some experiments.

Applying a nutritional stress during the differentiation process produced a pattern of changes in the growth-arrest gene mRNAs similar to those seen in the stem cells. The *gas2*, *gas5*, C/EBP- β and CHOP-10 mRNAs all underwent a transient increase within 24 h of transfer to the deficient medium. By 72 h, the mRNA levels had fallen and resembled those of cells in the control medium. The *gas3* and *gas6* mRNAs did not change for the first 24 h after treatment, but then increased 48–72 h after RA treatment. These changes were not due to a general change in mRNA levels as *g3pdh* levels were unchanged during differentiation in the control and lysine-deficient media; however, the low-serum medium caused an increase of approx. 70 % after 3 days.

DISCUSSION

Amino acid deficiency is much more effective than serum depletion in increasing the expression of the growth-arrest genes in F9 embryonal carcinoma cells, despite the fact that the *gas* family of genes was originally isolated from serum-deprived cells. These mRNAs are not simply elevated in growth-arrested cells but are overexpressed in a sequence, representing early and late events in the response to growth arrest. This ordered pattern of expression persists in differentiating cells and may be the result of amino acid deficiency activating one or more cell-cycle checkpoints [33,34]. However, the change in expression pattern of *gas2* and *gas3* shows that the cell phenotype and its stage of differentiation also plays a part in regulating the expression of individual growth-arrest genes.

The synthesis of *gas3* [15] and *gas6* proteins [6] in density-arrested serum-starved 3T3 cells correlates with increased mRNA levels. However, there is some evidence that protein levels do not always follow those of the mRNA and that translational regulation is important in the control of the growth-arrest genes [35,36]. It is possible that the increase in mRNA levels caused by amino acid deficiency is simply a homeostatic change to maintain the rate of translation when amino acid levels are limiting and may not lead to a corresponding change in protein levels. This is not the case for CHOP-10, where Bruhat et al. [37] have recently shown that the increase in mRNA in response to amino acid limitation in HeLa, HepG2 and Caco-2 cells leads to a corresponding increase in protein. Global protein synthesis was not reduced when cells were incubated in 70 μ M leucine. However, the levels of CHOP-10 protein were significantly increased. These results suggest that the sequential increases in mRNA levels at physiological amino acid concentrations are not simply a response to the inhibition of protein synthesis but represent mechanisms that can regulate growth and development in response to the available amino acid supply.

Our results show that lack of an essential nutrient such as lysine does not affect the differentiation of F9 embryonal carcinoma cells. Once the cells are committed to the differentiation pathway, it is then possible for them to complete all the steps that lead to the terminally differentiated phenotype. Culture

of F9 cells in serum- or amino-acid-deficient media does not induce the expression of the HoxB2, collagen type IV or laminin B2 genes without RA stimulation. The small increase in ALP can probably be explained by changes in the attachment of the cells to the extracellular matrix (see below). Only the mRNA for C/EBP- β is increased and it is likely that this is a stress response similar to that seen in fully differentiated tissues, for example during the acute-phase response in the adult liver [38–40]. C/EBP- β and CHOP-10 co-regulate each other [41] and respond to a variety of metabolic insults, including glucose [42] and amino acid deficiency [43]. There is no evidence that elevated levels of C/EBP- β mRNA lead to the expression of HoxB2, collagen type IV or laminin, and suggests that the ability of C/EBP- β to induce differentiation in adipocytes [18] is dependent on other factors that are not present in F9 cells. This additional factor may be responsible for the instability of other EC cell lines that must be kept in the exponential growth phase to prevent spontaneous differentiation or the induction of apoptosis [9].

There appear to be two independent mechanisms regulating ALP activity in F9 cells, one associated with differentiation, the other with changed cell–extracellular matrix interactions. The ALP activity of F9 cells depends upon the substratum, increasing by up to 4 times when the cells are grown on fibronectin or collagen type IV rather than gelatin [44]. Stimulation with RA produces a further increase in ALP, regardless of the substratum or the nutritional status of the cells. The increase in ALP in growth-arrested cells probably reflects a change in the interaction of the cells with the extracellular matrix, which leads eventually to detachment of the cells from the substratum and the induction of apoptosis [45,46]. The increased expression of the *gas2*, *gas3* and *gas6* genes in the growth-arrested cells suggests that they may be involved in nutritional regulation of stem-cell death. Neither the *gas2* nor the *gas3* gene product is lethal on its own, both proteins requiring other cell components before apoptosis can be initiated [14,15]. Increased levels of *gas2* and *gas3* may provide a means of priming growth-arrested cells for a further signal that triggers apoptosis. In the developing embryo this provides a mechanism for culling an overabundant population of stem cells. The over-expression of *gas6* during differentiation would support the idea that it too has a role in the regulation of apoptosis possibly counterbalancing the effects of *gas2* and *gas3*.

These results show that the C/EBP- β , *gadd153*/CHOP-10, *gas2*, *gas3*, *gas5* and *gas6* genes all respond to amino acid deficiency and that changes in mRNA levels are effective markers of nutritional stress in a rapidly dividing cell line. Studies both *in vitro* and *in vivo* suggest that the processes leading to the differentiation of embryonal carcinoma cells are highly analogous to those taking place in the developing embryo. It is likely that these mechanisms responding to nutritional stress are not confined to cell culture but are also operative during fetal development. Changes in the mRNA levels may be useful as markers of stem cell distress in the developing embryo, and also the growth-arrest genes may themselves be part of important mechanisms that change fetal development in response to the nutritional status of the mother.

This work was supported by the Scottish Office Agriculture, Environment and Fisheries Department. We thank Ms. A. Newman for operating the DNA sequencer.

REFERENCES

- 1 Barker, D. J. P., Gluckman, P. D., Godfrey, K. M., Harding, J. E., Owens, J. A. and Robinson, J. S. (1993) Lancet **341**, 938–941
- 2 Desai, M., Crowther, N. J., Ozanne, S. E., Lucas, A. and Hales, C. N. (1995) Biochem. Soc. Trans. **23**, 331–335
- 3 Schneider, C., King, R. M. and Phillipson, L. (1988) Cell **54**, 787–793

- 4 Fornace, A. J., Nebert, D. W., Hollander, M. C., Leuthy, J. D., Papathanasiou, M., Fargnoli, J. and Holbrook, N. J. (1989) *Mol. Cell. Biol.* **9**, 4196–4203
- 5 Fleming, J. V., Fontanier, N., Harries, D. N. and Rees, W. D. (1997) *Mol. Reprod. Dev.* **48**, 310–316
- 6 Manfioletti, G., Brancolini, C., Avanzi, G. and Schneider, C. (1993) *Mol. Cell. Biol.* **13**, 4976–4985
- 7 Coccia, E. M., Cicala, C., Charlesworth, A., Ciccarelli, C., Rossi, G. B., Phillipson, L. and Sorrentino, V. (1992) *Mol. Cell. Biol.* **12**, 3514–3521
- 8 Nakano, T., Kishino, J. and Arita, H. (1996) *FEBS Lett.* **387**, 75–77
- 9 Rudnicki, M. A. and McBurney, M. W. (1987) *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach* (Robertson, E. J., ed.), IRL Press Ltd., Oxford, U.K.
- 10 Alonso, A., Breuer, B., Steuer, B. and Fische, J. (1991) *Int. J. Dev. Biol.* **35**, 389–397
- 11 Strickland, S. and Mahadavi, V. (1978) *Cell* **15**, 393–403
- 12 Strickland, S., Smith, K. K. and Maroti, K. R. (1980) *Cell* **21**, 347–355
- 13 Wang, S.-Y. and Gudas, L. J. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 5880–5884
- 14 Brancolini, C., Benedetti, M. and Schneider, C. (1995) *EMBO J.* **14**, 5179–5190
- 15 Fabbretti, E., Edomi, P., Brancolini, C. and Schneider, C. (1995) *Genes Dev.* **9**, 1846–1856
- 16 Varnum, B. C., Young, C., Elliot, G., Garcia, A., Bartley, T. D., Fridell, Y.-W., Hunt, R. W., Trail, G., Clogston, C., Toso, R. J., et al. (1995) *Nature (London)* **373**, 623–626
- 17 Goruppi, S., Ruaro, E. and Schneider, C. (1996) *Oncogene* **12**, 471–480
- 18 Yeh, W., Cao, Z., Classon, M. and McKnight, S. L. (1995) *Genes Dev.* **9**, 168–181
- 19 Johnson, P. F., Landschulz, W. H., Graves, B. J. and McKnight, S. L. (1987) *Genes Dev.* **1**, 133–146
- 20 Landschulz, W. H., Johnson, P. F., Adashi, E. Y., Graves, B. J. and McKnight, S. L. (1988) *Genes Dev.* **2**, 786–800
- 21 Ron, D. and Habener, J. F. (1992) *Genes Dev.* **6**, 439–453
- 22 Saqib, K. M., Hay, S. M. and Rees, W. D. (1994) *Biochim. Biophys. Acta* **1219**, 398–404
- 23 Darrow, A. L., Rickles, R. J. and Strickland, S. (1990) *Methods Enzymol.* **190**, 110–117
- 24 Gilles, R. J., Didier, N. and Denton, M. (1986) *Anal. Biochem.* **159**, 109–113
- 25 West, D. C., Sattar, A. and Kumar, S. (1985) *Anal. Biochem.* **147**, 289–295
- 26 Gianni, M., Zanotta, S., Terao, M., Garattini, S. and Garattini, E. (1993) *Biochem. Biophys. Res. Commun.* **196**, 252–259
- 27 Chomczynski, P. and Sacchi, N. (1987) *Anal. Biochem.* **162**, 156–159
- 28 Jonk, L. J. C., DeJonge, M. E. J., Vervaart, J. M. A., Wissink, S. and Krijer, W. (1994) *Dev. Biol.* **161**, 604–614
- 29 Wood, L., Theriault, N. and Vogeli, G. (1988) *FEBS Lett.* **227**, 5–8
- 30 Oberbaumer, I. (1986) *Gene* **49**, 81–91
- 31 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
- 32 Papalopulu, N., Lovell-Badge, R. and Krumlauf, R. (1991) *Nucleic Acids Res.* **19**, 5497–5506
- 33 Gottlieb, T. M. and Oren, M. (1996) *Biochim. Biophys. Acta* **1287**, 77–102
- 34 Bartek, J., Bartkova, J. and Lukas, J. (1996) *Curr. Opin. Cell Biol.* **8**, 805–814
- 35 Manzow, S., Brancolini, C., Marks, F. and Richter, K. H. (1996) *Exp. Cell Res.* **224**, 200–203
- 36 Blais, S., Boudreau, F., Beaslieu, J.-F. and Asselin, C. (1995) *Dev. Dyn.* **204**, 66–76
- 37 Bruhat, A., Jousse, C., Wang, X.-Z., Ron, D., Ferrara, M. and Fafournoux, P. (1997) *J. Biol. Chem.* **272**, 17588–17593
- 38 Baumann, H., Morella, K. K., Campos, S. P., Cao, Z. and Jahreis, G. P. (1992) *J. Biol. Chem.* **267**, 19744–19751
- 39 Ray, A. and Ray, B. K. (1994) *Mol. Cell. Biol.* **14**, 4324–4332
- 40 Cao, Z., Umek, R. M. and McKnight, S. L. (1989) *Genes Dev.* **5**, 1538–1552
- 41 Fawcett, T. W., Eastman, H. B., Martindale, J. L. and Holbrook, N. J. (1996) *J. Biol. Chem.* **271**, 14285–14289
- 42 Carlson, S. G., Fawcett, T. W., Bartlett, J. D., Bernier, M. and Holbrook, N. J. (1993) *Mol. Cell. Biol.* **13**, 4736–4744
- 43 Marten, N. W., Burke, E. J., Hayden, J. M. and Straus, D. S. (1994) *FASEB J.* **8**, 538–544
- 44 Fleming, J. V., Hay, S. M. and Rees, W. D. (1995) *Biochem. Soc. Trans.* **24**, S221
- 45 Ruoslahti, E. and Reed, J. C. (1994) *Cell* **77**, 477–478
- 46 Langley, R. E., Palayoor, S. T., Coleman, C. N. and Bump, E. A. (1994) *Int. J. Radiat. Biol.* **65**, 605–610