British Journal of Nutrition (2005), **94**, 12–18
DOI: 10.1079/BJN20051447

The expression of growth-arrest genes in the liver and kidney of the protein-restricted rat fetus

Christopher A. Maloney, Christina Lilley, Morven Cruickshank, Caroline McKinnon, Susan M. Hay and William D. Rees*

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

(Received 16 September 2004 – Revised 15 December 2004 – Accepted 20 January 2005)

During fetal life, there are periods of rapid cell proliferation, which are uniquely sensitive to nutritional perturbation. Feeding the pregnant rat a protein-restricted diet alters the growth trajectory of major fetal organs such as the kidney. By day 21 of gestation, the ratio of kidney weight to total body weight is reduced in the fetuses of dams fed a protein-deficient diet. In contrast, the ratio of fetal liver weight to total body weight is unchanged. To investigate the mechanisms underlying this disproportionate change in organ growth in the low-protein group, cell proliferation and differentiation have been assessed in the liver and kidney. The steady-state levels of mRNA for the growth-arrest and DNA-damage gene *gadd153*/CHOP-10, CCAAT enhancer-binding proteins α and β were unaffected by maternal diet in both fetal liver and kidney. The mRNA for alpha-fetoprotein, albumin and hepatic glucokinase were unchanged in the liver, suggesting that maternal protein deficiency does not alter the state of differentiation. The steady-state levels of the mRNA coding for the cyclin-dependent protein kinase inhibitors ($p15^{INK4a}$, $p19^{INK4d}$, $p21^{CIP1}$, $p27^{KIP1}$ and $p57^{KIP2}$) were unchanged in the fetal livers but were significantly increased in the kidneys of fetuses from dams fed the low-protein diet. These results show that the asymmetrical growth of the kidney is associated with increases in mRNA for the Cip/Kip cyclin-dependent kinase inhibitors and that these may reflect specific lesions in organ development.

Fetal programming: Low-protein diet: Fetal origins of adult disease: Cell cycle: Organ development

Abnormalities of fetal and early postnatal growth are widely believed to be responsible for the association between low birth weight and the later development of hypertension and type 2 (non-insulin-dependent) diabetes mellitus in adulthood (Barker & Osmond, 1986; Hales & Barker, 1992). It is proposed that poor growth *in utero* permanently alters the morphology and physiology of specific organs such as the kidney (Gluckman & Hanson, 2004). These long-lasting changes in the relative sizes of the internal organs are produced by restrictions or imbalances in nutrient supply early in life (Widdowson, 1971). For example, a mild protein restriction alters the growth trajectories of key organs in the rat fetus, including the heart and kidney (Langley-Evans *et al.* 1996; Rees *et al.* 1999, 2000).

Changes in gene expression can be used to monitor differentiation and proliferation, and study the mechanisms that underlie this disturbance of the allometric relationships. For example, fetal liver cells express α-fetoprotein (AFP) (Nahon *et al.* 1988). Differentiation into the mature hepatocyte suppresses AFP expression and induces the expression of albumin (Liao *et al.* 1980) and hepatic glucokinase (Gruppuso *et al.* 1999). Cell differentiation is also regulated by the CCAAT enhancer-binding proteins (C/EBP) and the related *gadd153*/CHOP10 genes, which play a key role in integrating differentiation and nutritional status (Ramji & Foka, 2002). Cell proliferation is regulated at the level of the cell cycle by the activation and deactivation of a series of cyclin-dependent serine/threonine protein kinases (CDK). This positive regulation of the CDK system is counteracted by the action of two families of cyclin-dependent kinase inhibitors (CDKI). Numerous studies have shown that the CDKI proteins play an important role in the coordinate regulation of cell proliferation and differentiation during organ development (Westbury *et al.* 2001). In particular, both families of CDKI are expressed during prenatal hepatogenesis (Ilyin *et al.* 2003) and nephrogenesis (Terada *et al.* 1998). A number of pathological states associated with renal disease in adults also lead to increased CDKI expression in the kidney (Shankland & Wolf, 2000).

In the present paper, we report the effects of maternal protein deficiency on the mRNA for both CDKI and other markers of development in the liver and kidneys of the rat fetus. There are no alterations in the levels of mRNA associated with functional differentiation of the liver or kidney. There are specific changes in the mRNA for Cip/Kip CDKI in the fetal kidney, which may be associated with the asymmetrical growth of this organ.

Methods

Experimental diets

The experimental diets contained 180 g/kg or 90 g/kg protein and were similar to those previously described (Langley-Evans &

Abbreviations: AFP, α-fetoprotein; C/EBP, CCAAT enhancer-binding protein; CDK, cyclin-dependent kinase; CDKI, cyclin-dependent kinase inhibitor.

* **Corresponding author:** Dr William D. Rees, fax +44 (0) 1224 715349, email wdr@rri.sari.ac.uk

Jackson, 1996; Rees *et al.* 1999). The control, 180 g/kg protein, diet contained (g/kg) casein, 180; sucrose, 213; cellulose fibre (solkaflok) 50; corn starch, 425; vitamin AIN-76, 5; mineral mix AIN-76, 20; maize oil, 100; choline chloride, 2. The low-protein diets contained 90 g/kg casein, with compensating increases in the amounts (w/w) of sucrose and corn starch (1:3). Both the high- and low-protein diets were supplemented with 5 g/kg DL-methionine. Choline chloride and methionine were from Sigma Aldrich (Poole, Dorset, UK), and the other ingredients came from Special Diet Services (Witham, Essex, UK).

Animals

Female rats of the Rowett Hooded strain were fed the experimental diets commencing at 6–7 weeks of age. Two weeks later, when weighing approximately 230–240 g, the animals were mated with males of the same strain. Mating was confirmed by the detection of a vaginal plug, and this day was denoted day 0. The female rats were maintained on the same diets until day 21 of pregnancy.

Adult rats were killed by concussion followed by cervical dislocation; the fetuses were weighed and killed by decapitation. Maternal body weight was recorded after removal of the entire uterus and contents. The liver, kidney and heart of eight fetuses, chosen from each mother at random, were rapidly dissected and weighed. Samples were rapidly frozen in liquid N_2 and subsequently stored at -70°C. All experimental procedures were approved and conducted in accordance with the UK Animals (Scientific Procedures) Act 1986.

DNA extraction from fetal tissues

Approximately 50 mg frozen liver was removed from the liquid N_2 and crushed between two aluminium blocks cooled on solid CO_2. DNA was extracted from the frozen tissue using a Nucleospin DNA extraction kit in accordance with the manufacturer's instructions (Abgene, Epsom, Surrey, UK). Samples of 10 ng DNA were amplified with Taq DNA polymerase (Promega, Southampton, Hampshire, UK) using 1 nmol each of the Sry and G3PDH primers shown in Table 1 (Sakamoto *et al.* 2002). The PCR reaction comprised 25 cycles of 30 s at 94°C; 30 s at 55°C and 45 s at 72°C. Samples of the products were separated on a 1·5 % agarose gel (Flowgen, Ashby de la Zouch, Leicestershire, UK). Two bands of 144 bp (Sry) and 470 bp (G3PDH) were observed in male fetuses, whereas only a single band of 470 bp (G3PDH) was found in females.

RNA extraction from fetal tissues

Approximately 50 mg frozen liver or both kidneys from a single fetus were removed from the liquid N_2, and the frozen tissue was crushed between two aluminium blocks cooled on solid CO_2. The frozen, powdered tissue was transferred to a tube, and 0·5 ml Trizol reagent (Sigma Aldrich) was added immediately. The samples were disrupted for 2×10 s with an UltraTurrax homogenizer (Fischer Scientific, Loughborough, Leicestershire, UK) set at maximum speed. RNA was prepared by following the protocol supplied by the manufacturer, and the final pellet was dissolved in approximately 40 μl diethylpyrocarbonate-treated water. A sample of the RNA was checked for integrity and quantified using an Agilent 2100Bioanalyser (Agilent Technologies, Stockport, Cheshire, UK).

DNase treatment

Samples of 10 μg RNA were treated with 1 U RNAse-free DNAse (Promega) in 20 μl supplied buffer for 15 min at 25°C. The RNA was precipitated with ethanol, washed with cold 70 % ethanol and resuspended in 50 μl water. The RNA sample was reanalysed and quantified using the Agilent 2100Bioanalyser (Agilent Technologies).

Semi-quantitative real-time RT-PCR

Samples of RNA (50 ng) were reverse-transcribed in a final volume of 20 μl using TaqMan Reverse Transcription Reagents Kit (P/N N808-0234; Applied Biosystems, Warrington, Cheshire, UK). The reaction was primed with random hexamers at a final concentration of 2·5 μM. The reaction mixture was incubated at 25°C for 10 min and then at 48°C for 10 min. At the end of the reaction, the mixture was heated to 95°C for 5 min and then diluted with 80 μl water. A 2 μl sample of this mixture was diluted with a further 38 μl water ($20 \times$) to provide template for the 18S reaction.

The relative quantities of cDNA were measured using the SYBR Green real-time PCR kit (P/N 4309155; Applied Biosystems) following the protocol provided by the manufacturer. Triplicate 5 μl samples of the diluted reverse-transcription

Table 1. Sequences of primers used for PCR amplifications

Gene	Forward primer	Reverse primer
18S	CGGCTACCACATCCAAGGAA	GCTGGAATTACCGCGGCT
gadd153	CCACCACACCTGAAAGCAGAA	GAGGTAACCAAACAGGGAAACATAGT
p15	GGCTCAGAGACGAGCCCTGTA	AGATAGGGCTGGGGAGAAAA
p19	CTTCTTCACCGGGAGCTG	CACCAAAAGGGGTGAGAAAA
p21	CATGAGCGCATCGCAATC	TCCGCACAGGAGCAAAGTATG
p27	CCCGGTCAATCATGAAGAACTAA	GCTGGCTCGCTTCTTCCATA
p57	AGGAGCAGGACGAGAATCAA	TCTGGCCGTTAGCCTCTAAA
C/EBPα	TGCGCAAGAGCCGAGATAA	TGGTCAACTCCAACACCTTCTG
C/EBPβ	CTCTGATACCCGGACACCACTAC	TCTGTGGGTGAGTCACTTTGCT
α-fetoprotein	AGTGCTGCAAACTACCCACCAT	CGCCATTTTCGGCATGA
Hepatic glucokinase	AGTGTGCGCAGGCTGACA	CGCTGCCCTCCTCTGATTC
Sry	CCCGTGGAGAGAGGCGCAAGT	TAGGGTCTTCAGTCTCTCTGCGC
G3PDH	CCCTTATTGACCTCAACTACATGGT	GAGGGGCCATCCACAGTCTTCTG

mixture were used in 25 μl reactions. Similar reactions with primers specific for the 18S rRNA were set up containing 5 μl diluted cDNA sample. The reactions were followed for 40 cycles of PCR using an ABI Prism 7700 Sequence Detection System (Applied Biosystems) to determine the threshold cycle. Each plate also contained a standard curve comprising a serial dilution of pooled fetal liver or kidney cDNA to normalize the reactions. Relative target quantity was calculated from the standard curve, and the results were expressed as the ratio of the product relative to the product from the 18S rRNA.

Rat mRNA sequences were obtained from GenBank and primers designed using Primer Express Version 1.0 (Applied Biosystems). The sequences of the primers (MWG-Biotech, Milton Keynes, UK) used are given in Table 1. Ranges of primer concentration were tested to determine the optimum PCR yield. The products were separated on 3 % NuSieve agarose gels (Flowgen). All of the PCR products depended on prior treatment of the RNA with RT and produced single bands with no evidence of primer dimer formation. PCR products were cloned and sequenced to confirm their identity. Northern blotting using a cDNA probe for rat AFP was carried out as previously described (Fleming *et al.* 1998).

Western blotting

Samples of fetal serum were diluted 400-fold with PBS, and 5 μl aliquots were separated on 8 % SDS-polyacrylamide gels. The gels were blotted to Imobilon P membranes (Millipore, Watford, Hertfordshire, UK) and probed with a 1:1000 dilution of a goat polyclonal IgG anti-AFP antibody (Santa Cruz Biotechnology Inc., Heidelberg, Germany) and subsequently with a 1:10 000 dilution of anti-goat IgG conjugated to horseradish peroxidase (Sigma Aldrich). Enzyme-catalysed chemiluminescence was developed with the enzyme-catalysed chemiluminescence kit and detected with X-ray film (Amersham Biosciences Ltd, Chalfont St Giles, Buckinghamshire, UK). The films were scanned and the density of the bands quantified using Scion image. There was a linear relationship between absorbance units detected over a ten-fold range of serum volume loaded per gel lane.

Statistics

All data were analysed using the Genstat 6 statistical package (Lawes Agricultural Trust, Harpenden, Hertfordshire, UK). The data in Table 2 were analysed by ANOVA taking the dam into account in the block structure. Tissues from single fetuses were taken at random for mRNA estimation, and, to check that the samples chosen were representative of the population, the organ weight data were reanalysed by ANOVA using selection as a factor. This analysis showed that the samples chosen were not significantly different from those not chosen. The data for mRNA abundance in Figs 1 and 2 were analysed by the Mann–Whitney test as the values appeared not to be normally distributed.

Results

Animals were randomly assigned into two groups with mean weights of 204·4 (SD 11·3 g) and 204·7 (SD11·3 g) and then fed the experimental diets for 2 weeks. At the end of this period, the body weights were 240·3 (SD 8·3 g) for the group fed the diet containing 180 g/kg protein and 238·1 (SD 11·9 g) for the group fed the diet containing 90 g/kg protein (eight for both groups). After mating with normal males, the dams continued to be fed the experimental diets. One animal in the group fed 180 g/kg protein was subsequently found not to be pregnant and was excluded. The weights of the dams on day 20 of gestation were 344·7 (SD 10·1 g) for the group fed the diet containing 180 g/kg protein and 336·3 (SD 21·3 g) for the group fed the diet containing 90 g/kg protein. These weights were not significantly different, and the diets did not cause a change in weight gain over the gestation period.

Table 2. Organ weights of day 21 fetuses

	18 % protein		9 % protein		
	Mean	SD	Mean	SD	*P*
Number of dams	7		8		
Number of fetuses	12·9	2·7	12·9	4·7	0·9925
Fetus weight (g)	4·5	0·5	4·0	0·6	0·0530
Liver (mg)	269·7	47·3	228·4	60·8	0·0870
Kidneys (mg)	42·8	9·1	34·2	8·0	0·0080
Heart (mg)	21·3	4·9	22·1	6·3	0·6250
Liver (% body weight)	5·968	0·927	5·603	0·919	0·3550
Kidneys (% body weight)	0·944	0·182	0·858	0·128	0·0110
Heart (% body weight)	0·468	0·087	0·557	0·139	0·0040

Six fetuses from each litter were dissected and weighed. The data were analysed by ANOVA blocking for dam.

On day 21 of gestation, the dams were killed and the fetuses recovered. The average weight of all the fetuses recovered was 4·528 (SD 0·591 g) (*n* 103) from the dams fed 180 g/kg protein and 3·809 (SD 0·588 g) (*n* 95) from the dams fed 90 g/kg protein. These weights were significantly different when analysed by ANOVA using dam as the block structure ($P=0·016$). The data were also analysed using number of fetuses per dam as covariate. This showed no significant effect of litter size on fetal growth. Six representative fetuses were taken from each litter; their weights, together with those of the major organs, are shown in Table 2. The weights of the sample group were not significantly different from those not chosen.

There was a significant asymmetry in the growth of the fetal organs as a result of changing the protein content of the maternal diet. Table 2 shows that the weight of the fetal kidneys was significantly reduced in the fetuses of dams fed the 90 g/kg protein diet. In contrast, there was a smaller decrease in the weight of the fetal liver, although this failed to achieve statistical significance, and there was no effect of the maternal diet on the weight of the fetal heart. When the organ weights were expressed as a percentage of the total fetal weight, there was a significant decrease in the relative size of the kidney, whereas the relative size of the heart was significantly increased, showing a clear asymmetry in the growth of these particular organs. There was no effect of the maternal diet on the relative size of the fetal liver. The overall result of the present experiment is very similar to those of previous studies using the same strain of rat and similar diets (Rees *et al.* 1999).

DNA samples were prepared from a random selection of fetuses, and the sex was determined using a PCR specific for the *Sry* gene (Sakamoto *et al.* 2002). The organ weights of these offspring (seventeen males and nineteen females) were

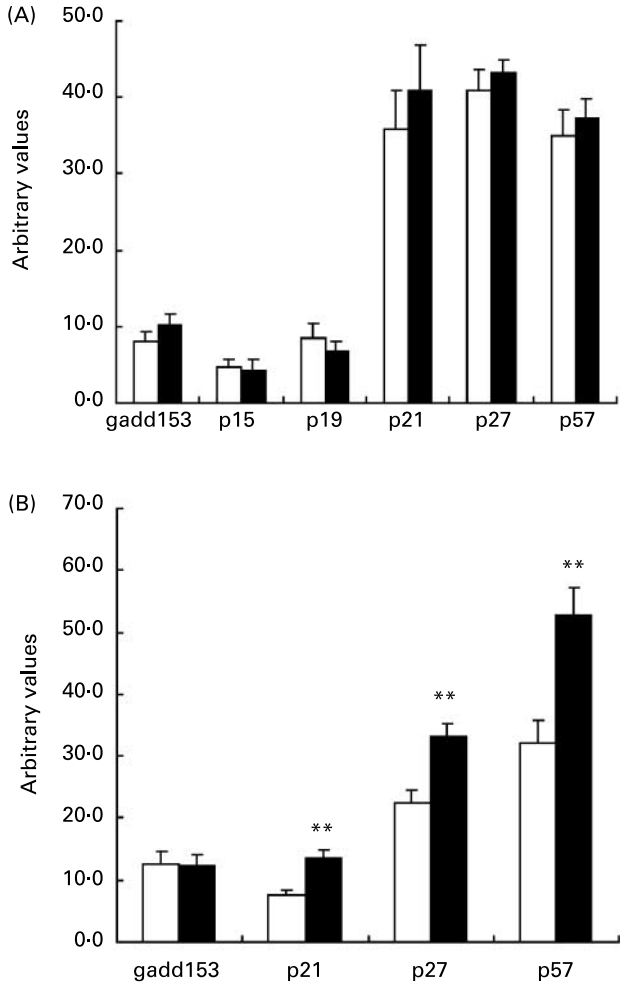

Fig. 1. The relative expression of messenger RNAs associated with cellular proliferation in the fetal liver (A) and kidney (B) of rat fetuses exposed to protein-deficient diets in utero. The open bars represent the fetuses of dams fed diets containing 18% protein, and the closed bars represent the fetuses of dams fed diets containing 9% protein. The data are shown as the ratio of the threshold cycle value for each complementary DNA normalized with respect to the 18S ribosomal subunit. There were six dams in each group, and tissues were taken from a single pup, chosen at random from the litter. Each reverse transcription reaction was carried out in triplicate. Error bars = SEM (n 6). $^{**}P<0{\cdot}01$.

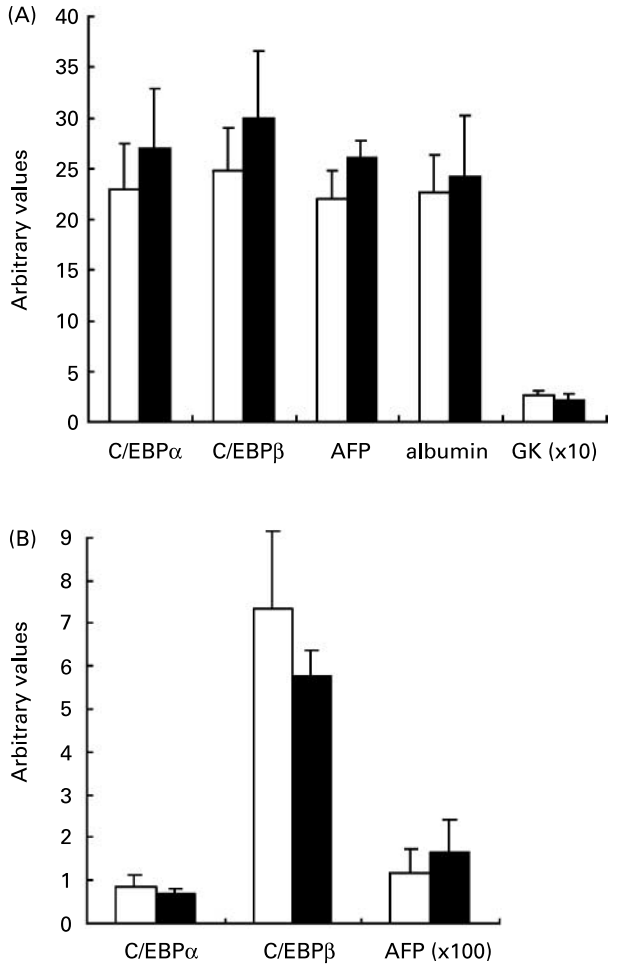

Fig. 2. The relative expression of messenger RNAs associated with the cellular differentiation of the fetal liver (A) and kidney (B) of rat fetuses exposed to protein-deficient diets in utero. The open bars represent the fetuses of dams fed diets containing 18% protein, and the closed bars represent the fetuses of dams fed diets containing 9% protein. The data are shown as the ratio of the threshold cycle value for each complementary DNA normalized with respect to the 18S ribosomal subunit. Values for hepatic glucokinase (GK) are $\times 10$. Values for kidney alpha-fetoprotein (AFP) are $\times 100$. There were six dams in each group, and tissues were taken from a single pup, chosen at random from the litter. Each reverse transcription reaction was carried out in triplicate. Error bars = SEM (n 6). C/EBP, CCAAT enhancer-binding protein.

then analysed by two-way ANOVA for the effects of maternal diet, fetal sex and diet–sex interactions. The results showed that the diet-dependent change in total growth was similar in both male and female fetuses, and there was no evidence for an interaction between the maternal diet and fetal sex. The asymmetrical development of the kidneys relative to the liver was observed in both male and female offspring.

Fig. 1(A) shows the relative expression of *gadd153* and the CDKI in the livers of day 21 fetuses from dams fed either the diet containing 180 g/kg casein or the diet containing 90 g/kg casein. There are no significant differences in the relative expression of any of these genes in the liver. In contrast, cDNA derived from fetal kidneys (Fig. 1(B)) showed significant increases in the expression of $p21^{CIP1}$, $p27^{KIP1}$ and $p57^{KIP2}$ when the protein content of the maternal diet was reduced. The maternal diet did not produce any change in the expression of *gadd153*/CHOP-10. Because of the low expression of $p15^{INK4a}$ and $p19^{INK4d}$ mRNA in the kidney samples, PCR products were only detected in some samples from both the high- and the low-protein groups. As a result, it was not possible to perform a quantitative analysis of these mRNA.

Semi-quantitative analysis of the cDNA from livers (Fig. 2(A)) and kidneys (Fig. 2(B)) of fetuses from dams fed either the high- or low-protein diet showed no significant differences in the expression of AFP, C/EBPα or C/EBPβ. There was also no effect of the maternal diet on the mRNA coding for albumin and hepatic glucokinase in the fetal liver. The levels of AFP mRNA were also measured independently by Northern blotting liver RNA samples and probing them with probes specific for rat AFP and 18S rRNA. The Northern blot data confirmed the result obtained with real-time PCR and also showed that the maternal diet did not significantly change the relative level of AFP mRNA in the fetal livers.

Because of the potential value of AFP as a marker of fetal growth retardation, the circulating levels of AFP were also measured in Western blots of fetal serum taken from the two groups of animals. Fig. 3(A) shows that an antibody specific for AFP detected a single band of 70 kDa, which is close to the predicted size for rat AFP (Liao *et al.* 1980). Quantification of the autoradiographs (Fig. 3(B)) showed that the protein content of the maternal diet did not significantly change the levels of AFP protein in fetal sera, although the levels were slightly more variable in the fetuses of the low-protein group.

Discussion

Numerous studies in laboratory rats have shown that a moderate reduction in the protein content of the maternal diet restricts growth and changes the relative sizes of the fetal organs (Snoeck *et al.* 1990; Langley & Jackson, 1994; Desai *et al.* 1995). These asymmetries in the growth of fetal organs are also observed with other metabolic disturbances such as those caused by gestational diabetes (Canavan & Goldspink, 1988). These data show that there are specific differences in mRNA level related to the disproportionate growth of the fetal liver and kidney.

The symmetrical change in the relative size of the liver induced by maternal protein deficiency does not affect the levels of mRNA for *gadd153*/CHOP10, the C/EBP transcriptional activators or for functional markers such as AFP, albumin and hepatic glucokinase. This is unlike the situation with agents that interfere with hepatic development, such as fenofibrate, which change these markers (Staels & Auwerx, 1992). The ratio of mRNA for functional proteins such as AFP or the C/EBP transcriptional activators are unchanged when expressed relative to constitutive RNA such as 18S rRNA, implying that cell function is normal with regard to the turnover of these RNA. This result suggests that each functional unit has a similar activity but that maternal dietary restriction reduces the number of these functional units, resulting in a smaller but otherwise normal liver. There is also evidence that maternal low-protein diets alter phosphenolpyruvate carboxykinase and glucokinase activity (Burns *et al.* 1997). There is, however, increasing evidence to suggest that these are indirect effects brought about by an altered endocrine environment as opposed to a disturbance in development (Bogdarina *et al.* 2004).

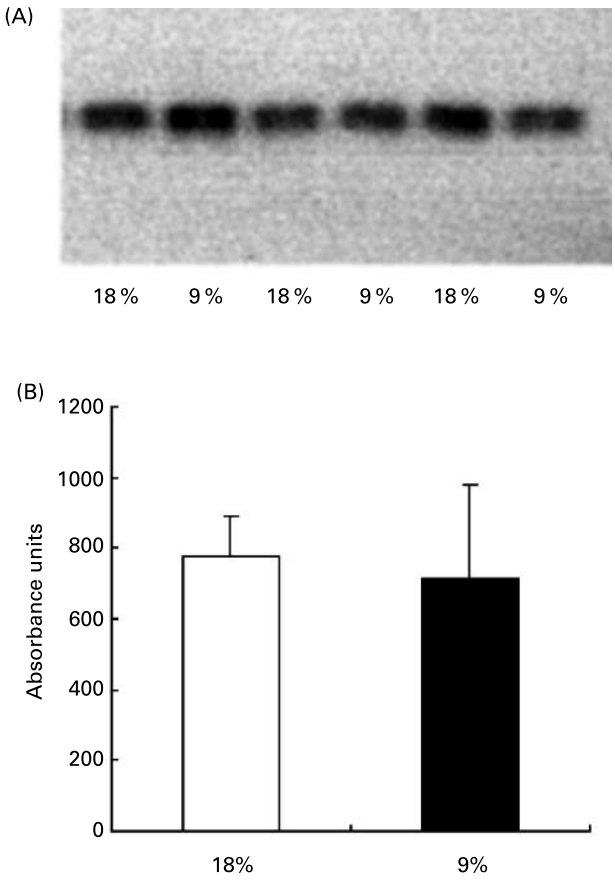

Fig. 3. (A) Samples of fetal serum separated on SDS-PAGE and blotted for (see Materials and Methods). 18 %, serum from the fetuses of dams fed diets containing 18 % protein; 9 %, serum from dams fed diets containing 9 % protein. (B) Quantification of the scanned blots. Error bars = SEM (n 6).

Feeding the protein-restricted diet to the dam causes a specific increase in the mRNA coding for $p21^{CIP1}$, $p27^{KIP1}$ and $p57^{KIP2}$ in the kidney. This is unlike the liver, where these markers are unchanged. At the same time, the mRNA for *gadd153*/CHOP10, the C/EBP isoforms and AFP are unaltered relative to 18S rRNA in the kidney. The changes in Cip/Kip mRNA levels may therefore be early markers for the change in structure shown to occur in the offspring of dams fed the low-protein diet. It is, however, not possible to interpret these results as simply demonstrating a reduction in cell proliferation. In the adult kidney, the expression of Cip/Kip proteins is associated with both hypertrophy and hyperplasia in pathological situations or in response to renal injury.

The Cip/Kip proteins bind to the cyclin–CDK complexes, inhibit their kinase activities and cause cell-cycle arrest (Kato *et al.* 1994; Sherr & Roberts, 1995). These genes are not essential for normal development, and studies of knockout mutants suggest that they play coordinating roles at developmental checkpoints, rather than having a direct involvement in cell commitment (Hiromura *et al.* 2001). For example, the size and organization of the kidney were normal in animals lacking $p57^{KIP2}$, but the inner medullary pyramid was smaller with fewer tubules and more stromal cells (Zhang *et al.* 1997). Studies of Cip/Kip expression in adult kidneys have shown that these genes play a vital role in the regulation of both glomerular and tubular cells (Shankland & Wolf, 2000). In the tubules, it appears that $p27^{KIP1}$ expression protects mesangial cells from apoptosis, and in glomerular diseases of the adult $p21^{CIP1}$ plays a multifactorial role in podocyte cell-cycle regulation (Shankland *et al.* 2000). These changes may also be related to important functional genes such as those for the angiotensin receptors, which are also influenced by maternal low-protein diets (McMullen *et al.* 2004). It is interesting to note that, *in vitro*, angiotensin II stimulates $p27^{KIP1}$ protein formation and stimulates tubulogenesis (Wolf *et al.* 2001).

Restricting the protein intake of the dam reduces the concentration of threonine and the branched chain amino acids in both the maternal and the fetal circulation (Rees *et al.* 1999). *In vitro* studies with embryonal carcinoma cells have shown that, when the availability of single amino acids falls below a critical level, the steady-state levels of the mRNA for the C/EBP isoforms and the related gene *gadd153*/CHOP10 are increased (Fleming *et al.* 1998). *In vitro*, amino acid deficiency also increases the steady state levels of the Cip/Kip mRNA, presumably as part of the same acute response to metabolic stress (Rees *et al.* 2003).

In vivo, however, maternal protein deficiency has no effect on the levels of *gadd153*/CHOP10 and C/EBP mRNA in either the liver or kidney. Furthermore, Cip/Kip mRNA levels are unchanged in the liver, suggesting that the restriction of maternal protein intake is not inducing the acute cellular response to amino acid deficiency.

All this evidence points to a more indirect mechanism of growth regulation, for example, growth factors such as insulin-like growth factor-2 or glucocorticoids (El Khattabi *et al.* 2003). Treating pregnant rats with glucocorticoids inhibits renal growth in their offspring, culminating in a lower nephron number, which has been suggested to lead to the development of hypertension. It has therefore been suggested that the low-protein diet interferes with the structural development of the kidney through an increase in fetal exposure to glucocorticoids (Langley-Evans, 1997). Glucocorticoids have been shown to increase the expression of $p21^{CIP1}$ in mesangial cells, through the action of a glucocorticoid response element in the promoter of the $p21^{CIP1}$ gene (Terada *et al.* 2001). Glucocorticoids are also known to inhibit the growth of cells (Seto *et al.* 1998). There is thus a possible link between the suggested role of glucocorticoids and changes in $p21^{CIP1}$ expression. However, the synthetic glucocorticoid dexamethasone administered to newborn rats also reduces AFP mRNA and the production of the protein (Huang *et al.* 1985). In our studies, we have not found significant changes in AFP in either liver or kidney, suggesting either that the AFP gene is less sensitive to glucocorticoids than the $p21^{CIP1}$ gene or that the changes in the kidney are mediated through another mechanism. Similarly, the lack of effect on $p21^{CIP1}$ expression in the liver suggests that if glucocorticoids are responsible for mediating these effects, there must be major differences in the sensitivity of the two tissues.

A number of studies in the rat have suggested that protein restriction during the period of active nephrogenesis late in gestation disrupts renal development and lowers nephron number. It has been suggested that this loss of renal function predisposes the offspring to the subsequent development of adult hypertension (Langley-Evans *et al.* 1999; Wintour *et al.* 2003; Woods *et al.* 2004). The current study shows a possible mechanism as changes in Cip/Kip mRNA levels may be associated with alterations in kidney morphology programmed at specific checkpoints in renal development.

Acknowledgements

This work was supported by European Union Fifth Framework programme NUTRIX (QLK1-2000-00 083) and the Scottish Executive, Environment and Rural Affairs Department as part of the Rowett Research Institute core funding. We wish to express our thanks to K. Simpson and D. Wallace for their skilled technical assistance.

References

Barker DJ & Osmond C (1986) Infant mortality, childhood nutrition, and ischaemic heart disease in England and Wales. *Lancet* **1**, 1077–1081.

Bogdarina I, Murphy HC, Burns SP & Clark AJ (2004) Investigation of the role of epigenetic modification of the rat glucokinase gene in fetal programming. *Life Sci* **74**, 1407–1415.

Burns SP, Desai M, Cohen RD, Hales CN, Iles RA, Germain JP, Going TC & Bailey RA (1997) Gluconeogenesis, glucose handling, and structural changes in livers of the adult offspring of rats partially deprived of protein during pregnancy and lactation. *J Clin Invest* **100**, 1768–1774.

Canavan JP & Goldspink DF (1988) Maternal diabetes in rats II. Effects on fetal growth and protein turnover. *Diabetes* **37**, 1671–1677.

Desai M, Crowther NJ, Ozanne SE, Lucas A & Hales CN (1995) Adult glucose and lipid metabolism may be programmed during fetal life. *Biochem Soc Trans* **23**, 331–335.

El Khattabi I, Gregoire F, Remacle C & Reusens B (2003) Isocaloric maternal low-protein diet alters IGF-I, IGFBPs, and hepatocyte proliferation in the fetal rat. *Am J Physiol Endocrinol Metab* **285**, E991–E1000.

Fleming JV, Hay SM, Harries DN & Rees WD (1998) Effects of nutrient deprivation and differentiation on the expression of growth-arrest genes (gas and gadd) in F9 embryonal carcinoma cells. *Biochem J* **330**, Part 1, 573–579.

Gluckman PD & Hanson MA (2004) The developmental origins of the metabolic syndrome. *Trends Endocrinol Metab* **15**, 183–187.

Gruppuso PA, Bienieki TC & Faris RA (1999) The relationship between differentiation and proliferation in late gestation fetal rat hepatocytes. *Pediatr Res* **46**, 14–19.

Hales CN & Barker DJ (1992) Type 2 (non-insulin-dependent) diabetes mellitus: the thrifty phenotype hypothesis. *Diabetologia* **35**, 595–601.

Hiromura K, Haseley LA, Zhang P, Monkawa T, Durvasula R, Petermann AT, Alpers CE, Mundel P & Shankland SJ (2001) Podocyte expression of the CDK-inhibitor p57 during development and disease. *Kidney Int* **60**, 2235–2246.

Huang DP, Cote GJ, Massari RJ & Chiu JF (1985) Dexamethasone inhibits alpha-fetoprotein gene transcription in neonatal rat liver and isolated nuclei. *Nucleic Acids Res* **13**, 3873–3890.

Ilyin GP, Glaise D, Gilot D, Baffet G & Guguen-Guillouzo C (2003) Regulation and role of p21 and p27 cyclin-dependent kinase inhibitors during hepatocyte differentiation and growth. *Am J Physiol Gastrointest Liver Physiol* **285**, G115–G127.

Kato JY, Matsuoka M, Polyak K, Massague J & Sherr CJ (1994) Cyclic AMP-induced G1 phase arrest mediated by an inhibitor (p27Kip1) of cyclin-dependent kinase 4 activation. *Cell* **79**, 487–496.

Langley SC & Jackson AA (1994) Increased systolic blood pressure in adult rats induced by fetal exposure to maternal low protein diets. *Clin Sci (Colch)* **86**, 217–222.

Langley-Evans SC (1997) Hypertension induced by foetal exposure to a maternal low-protein diet, in the rat, is prevented by pharmacological blockade of maternal glucocorticoid synthesis. *J Hypertens* **15**, 537–544.

Langley-Evans SC, Gardner DS & Jackson AA (1996) Association of disproportionate growth of fetal rats in late gestation with raised systolic blood pressure in later life. *J Reprod Fertil* **106**, 307–312.

Langley-Evans SC & Jackson AA (1996) Rats with hypertension induced by in utero exposure to maternal low-protein diets fail to increase blood pressure in response to a high salt intake. *Ann Nutr Metab* **40**, 1–9.

Langley-Evans SC, Welham SJ & Jackson AA (1999) Fetal exposure to a maternal low protein diet impairs nephrogenesis and promotes hypertension in the rat. *Life Sci* **64**, 965–974.

Liao WS, Conn AR & Taylor JM (1980) Changes in rat alpha 1-fetoprotein and albumin mRNA levels during fetal and neonatal development. *J Biol Chem* **255**, 10036–10039.

McMullen S, Gardner DS & Langley-Evans SC (2004) Prenatal programming of angiotensin II type 2 receptor expression in the rat. *Br J Nutr* **91**, 133–140.

Nahon JL, Tratner I, Poliard A, Presse F, Poiret M, Gal A, Sala-Trepat JM, Legres L, Feldmann G & Bernuau D (1988) Albumin and alpha-fetoprotein gene expression in various nonhepatic rat tissues. *J Biol Chem* **263**, 11436–11442.

Ramji DP & Foka P (2002) CCAAT/enhancer-binding proteins: structure, function and regulation. *Biochem J* **365**, 561–575.

Rees WD, Hay SM, Buchan V, Antipatis C & Palmer RM (1999) The effects of maternal protein restriction on the growth of the rat fetus and its amino acid supply. *Br J Nutr* **81**, 243–250.

Rees WD, Hay SM, Palmer RM & Antipatis C (2000) Maternal protein deficiency alters the fetal growth trajectory during late gestation. *Proc Nut Soc* **59**, 62A.

Rees WD, Prins ME & Hay SM (2003) Gene nutrient interactions as a result of amino acid deficiency; effect on mRNAs associated with the cell cycle. *EAAP Scientific Series:, Prog Res Energ Prot Metab* **109**, 69–72.

Sakamoto I, Takahashi T, Kakita A, Hayashi I, Majima M & Yamashina S (2002) Experimental study on hepatic reinnervation after orthotopic liver transplantation in rats. *J Hepatol* **37**, 814–823.

Seto M, Kim S, Yoshifusa H, Nakamura Y, Masuda T, Hamaguchi A, Yamanaka S & Iwao H (1998) Effects of prednisolone on glomerular signal transduction cascades in experimental glomerulonephritis. *J Am Soc Nephrol* **9**, 1367–1376.

Shankland SJ, Eitner F, Hudkins KL, Goodpaster T, D'Agati V & Alpers CE (2000) Differential expression of cyclin-dependent kinase inhibitors in human glomerular disease: role in podocyte proliferation and maturation. *Kidney Int* **58**, 674–683.

Shankland SJ & Wolf G (2000) Cell cycle regulatory proteins in renal disease: role in hypertrophy, proliferation, and apoptosis. *Am J Physiol Renal Physiol* **278**, F515–F529.

Sherr CJ & Roberts JM (1995) Inhibitors of mammalian G1 cyclin-dependent kinases. *Genes Dev* **9**, 1149–1163.

Snoeck A, Remacle C, Reusens B & Hoet JJ (1990) Effect of a low protein diet during pregnancy on the fetal rat endocrine pancreas. *Biol Neonate* **57**, 107–118.

Staels B & Auwerx J (1992) Perturbation of developmental gene expression in rat liver by fibric acid derivatives: lipoprotein lipase and alpha-fetoprotein as models. *Development* **115**, 1035–1043.

Terada Y, Inoshita S, Nakashima O, Kuwahara M, Sasaki S & Marumo F (1998) Cyclins and the cyclin-kinase system – their potential roles in nephrology. *Nephrol Dial Transplant* **13**, 1913–1916.

Terada Y, Okado T, Inoshita S, Hanada S, Kuwahara M, Sasaki S, Yamamoto T & Marumo F (2001) Glucocorticoids stimulate p21(CIP1) in mesangial cells and in anti-GBM glomerulonephritis. *Kidney Int* **59**, 1706–1716.

Westbury J, Watkins M, Ferguson-Smith AC & Smith J (2001) Dynamic temporal and spatial regulation of the cdk inhibitor p57(kip2) during embryo morphogenesis. *Mech Dev* **109**, 83–89.

Widdowson EM (1971) Intra-uterine growth retardation in the pig I. Organ size and cellular development at birth and after growth to maturity. *Biol Neonate* **19**, 329–340.

Wintour EM, Johnson K, Koukoulas I, Moritz K, Tersteeg M & Dodic M (2003) Programming the cardiovascular system, kidney and the brain – a review. *Placenta* **24**, Suppl A, S65–S71.

Wolf G, Wenzel U, Hannken T & Stahl RA (2001) Angiotensin II induces p27(Kip1) expression in renal tubules in vivo: role of reactive oxygen species. *J Mol Med* **79**, 382–389.

Woods LL, Weeks DA & Rasch R (2004) Programming of adult blood pressure by maternal protein restriction: role of nephrogenesis. *Kidney Int* **65**, 1339–1348.

Zhang P, Liegeois NJ, Wong C, *et al.* (1997) Altered cell differentiation and proliferation in mice lacking p57KIP2 indicates a role in Beckwith–Wiedemann syndrome. *Nature* **387**, 151–158.