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Partial sequences of two genes regulated by amino acid supply identified by the use of RNA fingerprinting by arbitrarily primed PCR

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RNA fingerprinting by random arbitrary primed PCR (RAP-PCR) using arbitrary 10 mer oligonucleotide primers was used to obtain partial sequences of two growth sensitive genes expressed in F9 embryonal carcinoma cells. The genes have been designated E800 and E550. The steady state levels of E800 and E550 mRNA increase when cells are cultured in lysine deficient medium. In F9 cells the pattern of E800 and E550 expression is similar to that of the growth arrest specific gene gas5. The partial sequence of E800 shows significant sequence identity to mouse and human expressed sequence tags. The deduced protein sequence shows some similarity to the ribosomal protein S7. The partial sequence of E550 shows no significant similarity with any entry in the database. In adult mice E800 is expressed in brain heart kidney adipose tissue and skeletal muscle and has a similar pattern of expression to the growth arrest gene gas5 in differentiated tissue. Our data suggest that the E800 and gas5 genes may share a common regulatory mechanism. (J. Nutr. Biochem. 9:136–141, 1998) © *Elsevier Science Inc. 1998*

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Introduction

Poor maternal nutrition has adverse effects on the development of the fetus, for example protein-calorie malnutrition leads to poor growth, low birthweight and an increased risk of disease in later life.[1,2] Protein deficiency in utero is linked with altered hepatic development and post-natal changes in protein and carbohydrate metabolism.[3–5] The rapidly dividing cells of the developing embryo have a high requirement for nutrients such as the essential amino acids and a deficiency in just one of these overrides other mitogenic factors and suppresses the growth rate. There is evidence that this growth retardation is not uniform and some organs such as the brain are protected, continuing to develop normally at the expense of other tissues such as the liver.[5] As a consequence, the offspring of the mother fed a protein deficient diet undergoes asymmetric growth and development. By identifying genes sensitive to nutritional stress and following changes in their expression, it may be possible to identify cells or tissues with different sensitivities to inadequate nutrition in utero.

The developing embryo is a complex array of different cell types that are difficult to manipulate in vitro. Tissue culture models offer a simpler system for the identification of genes regulated by nutrient deprivation. The mouse embryonal carcinoma cell line F9 has been used to identify many of the genes associated with growth and differentiation in the embryo. Embryonal carcinoma cells are the undifferentiated stem cells of teratocarcinomas and have many of the properties of pluripotent embryonic cells. Treatment with all-*trans*-retinoic acid causes the cells to differentiate into primitive endoderm, with an associated expression of gene markers for the differentiated phenotype.[6,7] However there is little information on the effects of nutrient deficiency on the growth and differentiation of these cells.

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Genes that are specifically overexpressed in growth arrested cells have been isolated by subtractive hybridisation of cDNA isolated from control and growth-arrested 3T3, CHO and hepatoma cells.[8,9,25] Recently the process of identifying differentially expressed genes has been simplified by the introduction of RNA fingerprinting using the random arbitrarily primed polymerase chain reaction (RAP-PCR).[10] RAP-PCR and the related technique of direct display PCR[11] uses random arbitrary 10 to 12 mer primers to amplify a representative selection of the mRNAs present in a cDNA preparation. By comparing the arrays from cells cultured under different conditions differentially expressed genes can be identified. This technique has been used successfully to isolate PCR fragments of differentially expressed genes from a number of different sources.[12–14] This paper reports the application of this technique to embryonic cells and the identification of two new mRNA transcripts that are elevated in growth arrested cells.

Methods and materials

Cell culture

Mouse F 9 embryonal carcinoma cells (American Type Culture Collection, Rockville, MD, CRL-1720) were cultured in Dulbeccos Modified Eagle's essential medium containing 10% fetal calf serum (Gibco/BRL, Paisley, Scotland). The medium was changed every 2 days.[15] Lysine deficient medium has been described previously.[16] 1.2×10^6 cells (control) and 2×10^6 cells (growth arrested) were seeded in 90mm gelatin coated dishes and after overnight culture in complete medium were transferred to test medium for 48 hr before harvesting. Growth rates were determined by staining fixed cells with crystal violet as described previously.[17]

Isolation of Poly (A+) RNA

Total RNA was isolated from cultures of F9 cells by the guanidine isothiocyanate-phenol method.[18] 250 to 300 μg of total RNA was dissolved in 200 μL of buffer containing: 500mM NaCl, 10 mM tris-Cl (pH 8.0), 1 mM EDTA, and 0.5% w/v SDS. The solution was heated to 65°C for 5 min and cooled on ice before 0.2 mL of oligo dT cellulose slurry (type 7 Pharmacia, Uppsala, Sweden). was added. The mixture was equilibrated for 3 h at room temperature, transferred to a miniature spin column, and washed four times with buffer containing 250 mM NaCl, 10 mM Tris-Cl (pH 7.4), and 1 mM EDTA. Poly (A+) RNA was eluted by washing the column three times with TE buffer [10 mM tris-Cl (pH 7.4), 1 mM EDTA], precipitated with ethanol and resuspended in DEPC-treated water. The recovery was estimated by measuring the absorbance at 260 nm, and was about 5% of the total RNA applied to the column.

Reverse Transcriptase PCR

One μg of poly (A^+) RNA was heated to 70°C for 10 min with 40 ng of random hexamer primers (Genosys Biotechnologies Inc, The Woodlands, TX) then placed on ice for 10 min. The reverse transcription was carried out in a 20 μL volume containing: 50mM Tris-Cl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 200 μM of each dNTP, and 200U Superscript reverse transcriptase (Gibco). The reaction mixture was incubated at 37°C for 1 hr before being heated to 95°C for 5 min to terminate the reaction.

The reverse transcription mixture was used as template for PCR with the arbitrary primers (Restriction site set GEN 1-RE, Genosys). The PCR reaction was performed in a 20 μL volume of buffer containing 2.5 μM primers, 200 μM of each dNTP, 2 mM $MgCl_2$, 100 ng cDNA template (2 μL of the RT mix), and 2.5 U of Taq DNA polymerase (Gibco). cDNA samples were denatured by heating to 93°C for 5min, followed by 40 cycles of PCR comprising denaturation at 93°C for 30 sec, annealing at 50°C for 30 sec (except for the first cycle, which was 45°C for 5 min) and extension at 72°C for 30 sec. Products were fractionated on a 1.2% agarose gel, stained with ethidium bromide and the products visualized on a transilluminator.

Cloning and sequencing

Bands of interest were cut out of the gel with a clean sterile scalpel blade. The gel slice was frozen to disrupt the gel, thawed and the agarose removed by filtration through a 0.2 μ centrifuge filter (Spin-X, Costar, Cambridge, MA). The filtrate was used as template to re-amplify the product using Pfu DNA polymerase (Stratagene, LaJolla, CA). The products were subcloned into the vector pCR-Script SK(+) using the SrfI cloning kit (Stratagene) and transfected into E.coli XL1Blue. Plasmid was purified using Wizard mini-prep kits (Promega, Madison, WI). Products were sequenced in both strands with an ABI 373A automatic DNA sequencer using the Taq-Dye-deoxy terminator kit (Applied Biosystems Inc, Foster City, CA).

Northern analysis

Twenty μg of total RNA isolated as described previously was separated on a 1.2% agarose gel. The gel was stained with ethidium bromide to confirm that equal amounts had been loaded before transfer to a nylon membrane (Boehringer, Lewes, East Sussex, UK). Probe templates were prepared by EcoRI digestion of the plasmid and labelled with [α-^{32}P]-dCTP using a Megaprime labelling kit (Amersham, International Little Chalfont, Bucks, UK). Isolation of the gas5 probe has been described previously.[28] Hybridisations were performed according to standard protocols[19] and washed to high stringency in $0.1 \times$ SSC + 1% SDS at 65°C. Blots were imaged on a wire proportional counter (Packard Instant Imager). Using the imager software, boxes of the same size were drawn around the bands on the Northern blot and the counts recorded during the acquisition period were quantified. The results are expressed as a percentage of the signal derived from exponentially growing cells. The specific activities of the probes were calculated from standard samples of plasmid DNA applied to the nylon membrane and hybridized with the Northern blots.

Results

Complete Dulbeccos minimal essential medium used for the culture of F9 cells contains 10% fetal calf serum and approximately double the physiological concentrations of each of the essential amino acids. Growth of F9 cells was arrested by culturing them in a medium deficient in L-lysine. Cells were subcultured from exponentially growing cultures and grown overnight in complete medium before transfer to a medium containing 10% fetal calf serum but deficient in L-lysine. The lysine deficient medium had a free L-lysine content of 19 nmol/mL compared with 200 nmol/mL in the complete medium. To compare growth rates in the two media F9 cells cultured for 42 hr were stained with crystal violet. The optical density increased by 3.2-fold in cells cultured in complete medium compared with 1.2 times in the deficient medium. The estimated doubling time in the deficient medium is at least 10 times longer than in the complete medium, although some growth had still taken

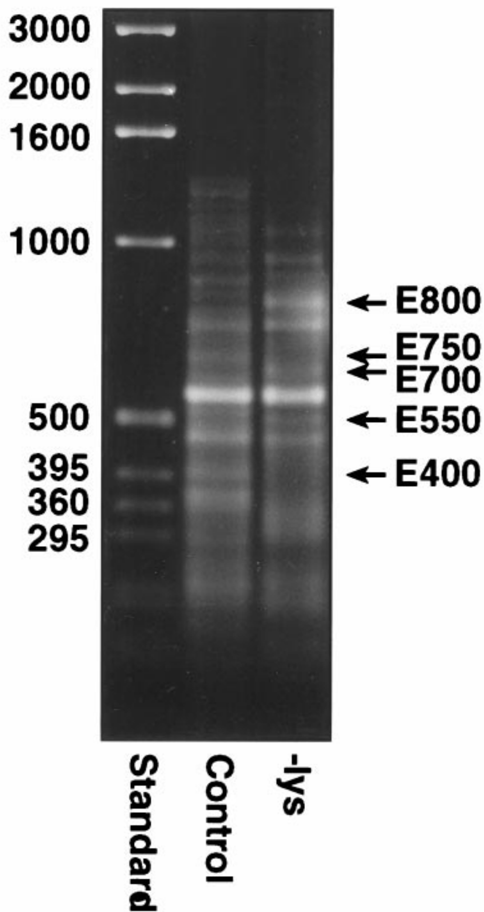

Figure 1 Electrophoresis of RAP-PCR products isolated from F9 embryonal carcinoma cells. cDNA derived from cells cultured in complete or lysine deficient media was amplified with an arbitrary primer of sequence 5′-GCGAATTCCG-3′. Standard, 1-kb ladder (Gibco); Control, cells grown in complete medium; -lys, cells grown in lysine deficient medium. The locations of the bands taken for further study are shown with an arrow.

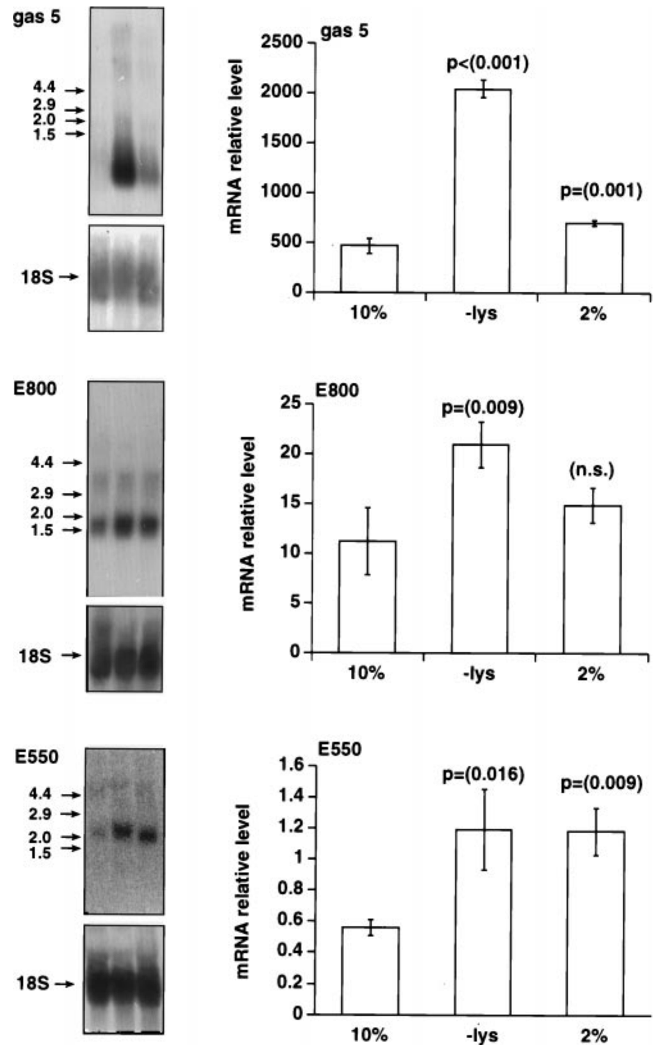

Figure 2 Northern analysis of F9 RNA probed for: gas5; E800; and E550. The left hand side shows an autoradiograph of a representative blot of 20 μg total RNA. The treatments were (left to right). cells cultured in complete medium (10%), lysine deficient medium (-lys)., and complete medium containing 2% newborn calf serum (2%). The arrows indicate the locations of RNA standards and their size in bases. Loading was corrected by measuring the hybridisation of a probe for the 18S ribosomal RNA shown in the lower panel. The film was deliberately overdeveloped to show the minor bands. A quantitative analysis of the probe hybridisation to the major transcript determined using the proportional wire counter is shown in the histogram in the right hand side. Data are the average from four experiments. Error bar = SEM ($n = 4$), n.s = not significant.

place. A 48-hr period of culture in the deficient medium caused no loss of viability and the cells grew normally when re-fed with complete medium.

cDNA was prepared from poly (A^+) RNA isolated from exponentially growing F9 cells and from cells grown in lysine-deficient medium. Equal amounts of the cDNA preparations were used as templates for random arbitrary primed PCR using a series of arbitrary decamer primers that corresponded to the recognition sequences of a variety of common restriction enzymes. Each of the 10 primers tested produced quite distinct patterns, where most of the major bands were common to the exponentially dividing and growth-arrested cells. The patterns of the major products were quite reproducible with mRNA prepared from three batches of cells giving essentially identical results. In addition there were also a number of minor bands, some of which seemed to be differentially expressed. A typical result for polymerase chain reactions using a primer that encodes the EcoRI recognition sequence is shown in *Figure 1*. Five bands that appeared to be differentially expressed in growth arrested cells were chosen for further investigation (shown by arrows in *Figure 1*). The DNA was isolated from the gel, reamplified by PCR, and subcloned into a plasmid vector. The sizes of the resulting cloned cDNAs were consistent with the sizes of the bands from the original gel.

Because these PCR techniques frequently produce false positives, the cloned cDNAs were used as probes for Northern blotting to confirm that the PCR products correspond to cellular mRNAs that are regulated by growth arrest. The steady state level of the growth arrest specific gene 5 (gas5) transcript (*Figure 2* top panel) was increased by about 4-fold when F9 cells were transferred from complete to lysine-deficient medium. This is similar to the

A

E800

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  1  GAATTCCGGG AGCCCTTGAT TGACAAGGAA TATTACCGCA AGCCGGTGGC
 51  GGGAACTCAC CGAAGAGGAG AAGTATGACC AGGAGCTCAA GAAAACCCAG
101  TTTATCAAAG CTGCGGCCGC GACTGAAACC AGCTCGGTGT TCGCAGACCC
151  GGTCATCAGC AAATTCACCA ACATGACGAT GAAGGGAGGC AACAAAGTTC
201  TGGCCCGGTC TCTCATGGCT CAGACTCTGG AAGCTGTGAA AAGGAAGCAG
251  TTTGAAAAGT ACCGTGCTGC CTCTGCGGAG GGAACAGGCC ACCATTTGAA
301  CGGAACCCCT ACCAGGATCT TCCACGAGGG CACTCAAAAA CTGTGTAGCC
351  TGTGGATTGG GTTGGTGCCG ATCCTCAAAG GGGGCCATTT CTACCAGGTC
401  CCCGTGCCTC TCGGTGTACC GACGGTCGGC GCTTCTTGGG CATGAAGTGG
451  GATGATCACA GAGTGCCGAG AGAATAAACG GCGGCGGACA ATTATGCCAG
501  AGGGAACTTT TCAAATGAGC TGCTGGAGGC TTTTCATAAC CGAGGTCCCG
551  TGATCAAGAG GAAGCACAAT ATGCATAAGA TGGCAGAGGC CAACCGTGCC
601  CTGGCCCACT ACCGCTGGTG GTAGCAGGAG AGGAGAGGTG GGAAAGGACA
651  CACTCCCTCG AGAAACCACC CGACGGCTGG AGAGGTGGCT CAGTGGTTAA
701  AGCCACTCAC TGCTCTTTCC ACAGGACCTG GTTTGATTCC CAGCACTCAC
751  ACGGAGGCTC GCTGACCACC TGTAACTCCT GTTCCAAAGG TTTCGATACC
801  TTCTGACCTC TGGGCGGAAT TCGC
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E550

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  1  CGAATTCCGG AGGACACCCA TGTAGCTCCG TGGTAGTGCT ATGATCCCGC
 51  CACAGGTTGC TGGAGCTTCG TGGCGGACTT GCCACAGCCG GCTACAGGGG
101  TTCCCTGTGC CCAGGCACGT GGCCGCCTCT TCGTGTGCCT GTGGAGGCCG
151  GCAGACATCA CTGCCGTTGT CGAGTAGTGG TGCAAATGGA CAAGTGGGCT
201  GCCCAGTGGC TGAACTGTGT CGTTCACAGA CGTACGGTCA CTTTATGGTG
251  GCCCATCGCG ATATTCTCTA TGTAGTACGA AATGGACCTT CCGATGATTT
301  CTGCACTGTG CCATCGATTG CCTCAACCTG GTCACAGGCC AGTGGACATC
351  GTTACCTGGC CAATTTGTCA ACAGCAAGGG AGCACTCTTC ACGTCTGTAG
401  TGCGTGGGGA CACCGTTTAT ACTGTCACCC GTATATCCAC GTTGGTCTAT
451  GCCATTGAAG ATGGCACCTG GCGGCTGCTC AGGGAAAAGG CTGGCTTTCC
501  TCGGAATTCG C
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B

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               E800 :   511 SHELLEAFHNXGPVIKRKHNMHKMAEANRALAHYRW
                            + E++ A++N G  IK+K + H+MAEANRA AHYRW
Ribosomal protein S7:    73 AEEIIAAYNNTGTAIKKKEDTHRMAEANRAFAHYRW
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Figure 3 A, Sequences of PCR fragments derived from E800 and E550 genes. B, Protein sequence similarity shared by E800 and ribosomal protein S7 identified by BLAST searching of GenBank database.

increase described previously in growth-arrested Friend leukaemia (FL) and NIH 3T3 cells[8,20] and confirmed that culture in the lysine-deficient medium had induced a recognised growth arrest gene. The blots were sequentially probed with the five products isolated from the RAP-PCR experiment. All the RAP-PCR products hybridized to mRNA from F9 cells, showing them to be representative of cellular RNAs. Two transcripts, E800 and E550 were both increased in growth arrested cells (*Figure 2*, middle and lower panels). There were no significant changes in the expression of the remaining three transcripts, which were assumed to be false positives.

The E800 probe hybridized to two transcripts, a major transcript of 1450 bases and a minor transcript of 3900 bases. There were also two E550 transcripts of 1800 bases and 4100 bases. The 1.8-fold increase in E800 was specific for amino acid deprivation and quiescent cells in low serum medium did not show any significant increase in the mRNA level. In contrast, E550 was increased by about 2-fold in both amino acid and serum deprived cells. Culture at a higher cell density, when the cells were close to confluence, increased the expression of E800, E550 and gas5 by two to three fold and abolished the effect of amino acid deprivation. It was important to ensure that the cells in complete medium were sampled during the exponential phase of growth, as the levels of all three gene transcripts rose after 2 days, apparently as an early consequence of the culture medium becoming exhausted. This increase was reversed by the addition of fresh serum (data not shown).

The inserts from the plasmids containing the E800 and E550 PCR products were both sequenced (*Figure 3*). The sequences of the two inserts, which were both bounded by

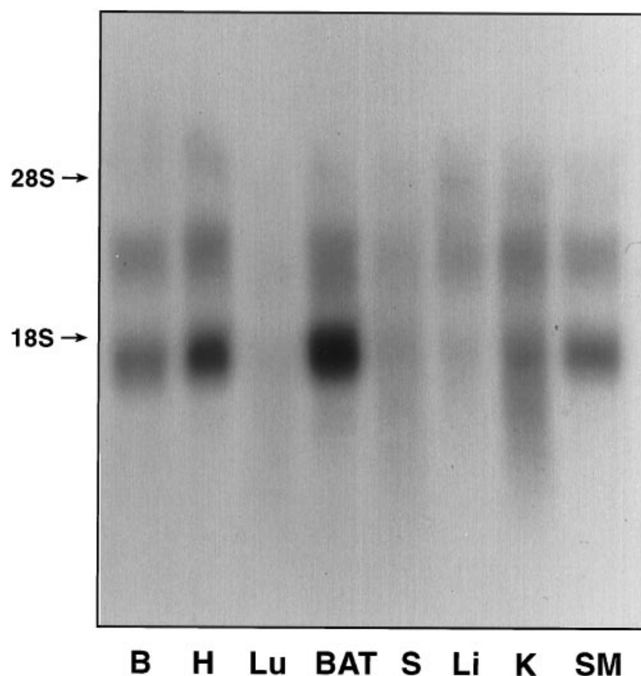

Figure 4 Tissue distribution of E800. 10 μg of total RNA was separated on an agarose gel and probed for E800 gene expression. The tissues used were brain (B), heart (H), lung (Lu), brown adipose tissue (BAT), spleen (S), liver (Li), kidney (K), and skeletal muscle (SM). The arrows indicate the location of the 28S and 18S ribosomal subunits.

the primer, were compared with the GenBank sequence database using the FASTA program. E550 showed no significant similarity with any entry in the database. E800 was similar to two expressed sequence tags, one isolated from a mouse embryo (Accession No. W30112; with a 94% similarity over a 506 bp overlap) and the other from the brain of a human infant (Accession No. T74441; with a 77% similarity over a 444 bp overlap).[21,22] The deduced protein sequences were compared with the GenBank database using the BLAST program.[23] The longest open reading frame in E800 contained a motif present in members of the prokaryotic S7 ribosomal protein family[24] as shown in *Figure 3B*. 20 of the 36 amino acids in this motif are similar (55%), and 77% are conserved.

Figure 4 shows the distribution of E800 expression in the tissues of an adult mouse. The gene was extensively expressed in most tissues. The highest levels were found in brain, heart, brown adipose tissue, kidney, and skeletal muscle with lower levels of expression in lung, spleen, and liver. E550 expression in adult tissues was much lower and the mRNA could not be detected by Northern analysis.

Discussion

We have isolated PCR fragments of two genes whose transcript levels are increased in cells where growth has been arrested by amino acid deprivation. These add to the list of genes that include transcriptional activators, growth factors, membrane proteins, and a variety of structural proteins.[29,30] One of the structural proteins, the mRNA for the ribosomal protein L22 is upregulated by 2 to 3-fold in amino acid starved hepatoma cells.[25,26] Because there is some sequence similarity between E800 and members of the ribosomal protein S7 family, it is tempting to speculate that the protein product of E800 codes for a hitherto unidentified mouse ribosomal protein. However, the partial nucleotide sequence of E800 was not related to any of the mouse ribosomal protein sequences present in the GenBank database and E800 cannot be assigned a function until the complete open reading frame has been identified.

E800 seems to be regulated in a very similar way to the growth arrest specific gene gas5. Both genes are more sensitive to nutrient-mediated growth arrest than to the removal of serum. The expression of gas5 in adult tissues is similar to E800 with high level expression in brain, heart, lung, and kidney and lower levels in liver and spleen.[20] This suggests that gas5 and E800 may share a common regulatory mechanism that is sensitive to the nutrient supply. When cells are deprived of nutrients mRNAs needed for the expansion of cell architecture such as ribosomes will be required for a correspondingly longer period. This can be achieved either through an increase in the rate of transcription or by stabilization of the message. Studies of the regulation of gas5 expression in 3T3 and FL cells[27,20] showed that multiple post-translational events regulate gas5 stability, although there was no evidence for a large increase in stability. It is more likely that prolonged culture in growth retarding media may lead to a partial synchronisation of the cells. A greater proportion of the arrested cells would be held up at a particular stage of the cell cycle when compared with a normal population. This would result in an apparent increase in the steady state transcript levels of genes required for specific cell cycle stages.

The E800 gene is represented in expressed sequence tags from a number of preimplantation and postimplantation embryonic cDNA libraries. Because this gene is expressed during embryogenisis it may be of use in identifying stem cell populations in the embryo that are affected by nutrient deficiency.

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